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Purification and properties of proteases produced by *Alternaria alternata* (Fr.) Keissl, regulation of production by fructose

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Abstract. A strain of *Alternaria alternata* (Fr.) Keissl, when grown on wheat bran Czapek Dox medium was found to secrete one neutral and two alkaline proteases. The purified enzymes were found to be endo peptidases, the alkaline proteases being serine proteases and neutral proteases being cysteine proteases. Fructose when added to the culture medium was found to give rise to a new neutral protease at the expense of the neutral protease produced in the absence of fructose and was also found to enhance the production of alkaline proteases. It also appears that fructose modifies the alkaline proteases with respect to some characteristics such as V_{max}, Ea etc. Sodium dodecyl sulphate polyacrylamide gel electrophoresis indicated a significantly altered protein profile in fructose supplemented medium.

Keywords. Alternaria; proteases; fructose.

Introduction

A strain of *Alternaria alternata* (Fr.) Keissl isolated in this laboratory from infected mandarin oranges (Kunte and Shastri, 1979) was found to produce proteolytic enzymes in wheat bran Czapek Dox medium (Patil and Shastri, 1981). A crude culture filtrate of *A. alternata* exhibited two broad shoulders of activity in the pH range of 6·0–7·5 and 8·0–9·5 which indicated the possible presence of more than one protease in the culture filtrate. Although, a number of *Alternaria* species have been shown to produce extracellular proteases, only a few of them such as those produced by *A. tenuissima* have been purified and characterized (Jonsson and Martin, 1969).

It was also reported by the authors (Patil and Shastri, 1982) that supplementation of normal wheat bran Czapek Dox medium with fructose led to a greater production of proteases in the culture medium. It was also seen that fructose does not affect the secretary properties of the membrane (Patil and Shastri, 1983). This indicated that fructose enhances the production of these enzymes. The effect of fructose could be either due to an increase in the production of one or more of these enzymes and/or due to the production of a new protease. Another possibility was that fructose might alter some of the properties of proteases which are reflected in greater than normal activities

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Abbreviations used: TPCK, N-Tosyl phenylethylchloro methyl ketone; TCA, trichloroacetic acid; SDS, of these enzymes. To elucidate these points, proteases produced in normal medium and in the presence of fructose were purified and compared. The present paper describes the results obtained.

Materials and methods

The organism *A. alternata* was maintained on potato dextrose agar slants. Transfers were made once a month. Casein Hammerstein quality was from Nutritional Biochemical Corporation, Cleveland, Ohio, USA; Sephadex G-75 and Sephadex G-100 were from Uppsala, Sweden, DEAE-cellulose, ferulic, caffeic and chlorogenic acid, all the di-, tri- and tetrapeptides, CBZ-derivatives of di-, tri- and tetrapeptides, dithionitrobenzoic acid, *p*-chloromercuribenzoate, phenylmethylsulphonylfluoride, N-tosyl phenylethylchloro methyl ketone (TPCK), iodoacetate, N-acetyl-L-tyrosine, N-acetyl-L-tyrosine ethyl ester, hippuric acid, hippuryl-L-arginine, hippuryl-L-phenylalanine, leu-*p*-nitroanilide, N-benzoyl-DL-arginine-*p*-nitroanilide, *p*-nitroaniline, sodium dodecyl sulphate and the molecular weight marker kit containing lysozyme, β-lactoglobulin, pepsin, trypsinogen, ovalbumin and bovine serum albumin were from Sigma Chemical Company, St. Louis, Missouri, USA. EDTA, β-mercaptoethanol, ammonium sulphate were of analytical reagent grade.

Buffers

Buffers were prepared by the method of Gomori (1955). Sodium phosphate buffer, 0·2 M, pH 6·0–8·0 and glycine-NaOH buffer, 0·2 M, pH 8·5–10·5 were used.

Preparation of culture filtrate

Wheat bran Czapek Dox medium was adjusted to pH 6·2 and sterilized at 120°C for 15 min. Fructose solution was adjusted to pH 6·2 and mixed with normal medium at 3 % level after sterilization. Inoculation of normal and fructose supplemented medium was carried out at 5 % level with spore suspension of organism prepared in sterile distilled water and adjusted to 2×10^6 spores/ml. Incubation was carried out at 26 ± 2°C under static conditions. After 5 days the contents of the flasks were filtered through Whatman No. 41 filter paper discs. Culture filtrates were centrifuged at 5000 *g* for 10 min and the supernatant was used as the crude enzyme.

Determination of protein

Protein content in crude and purified enzymes were measured by the method of Lowry *et al.* (1951). In crude enzymes obtained from fructose supplemented medium proteins were precipitated by trichloroacetic acid (TCA), dissolved in 0·1 N NaOH and then measured by the method of Lowry *et al.* (1951). Proteins in column effluents were also determined by measuring absorbance at 280 nm.

suitably diluted enzyme were incubated for 20 min at 37°C. Reaction was stopped by the addition of 3 ml of 5% TCA. The contents of the tubes were filtered through Whatman No. 3 after 30 min at room temperature and suitable aliquots of the filtrate were assayed for TCA soluble peptide fragments by the method of Lowry *et al.* (1951). Appropriate zero time controls were also run simultaneously. One unit of protease activity was defined as that amount of enzyme which liberates 1 mg equivalent of peptide fragments under the assay conditions using bovine serum albumin as standard. Specific activity is expressed as units per mg protein.

Peptidase activity

Peptidase activity of purified proteases using various peptides was determined by the method of Peter (1975).

Esterase activity

Esterase activity with N-acetyl-L-tyrosine ethyl ester was determined by the method described by Sudhakar Prabhu and Pattabiraman (1977). Liberated N-acetyl tyrosine was measured by the method of Lowry *et al.* (1951) using N-acetyl-L-tyrosine as a reference standard. One unit of esterase activity is defined as that amount of enzyme which liberates 1 mmol of N-acetyl tyrosine under the assay conditions.

Carboxypeptidase activity

Carboxypeptidase activity was measured by the method of Folk *et al.* (1960) using hippuryl-L-arginine and hippuryl-L-phenylalanine as substrates and hippuric acid as a standard.

Aminopeptidase activity

Aminopeptidase activity was determined by the method of Todanobu and Seeichi (1977) using Leu-gly-gly, DL-Leu-gly-gly, Leu-*p*-nitroanilide and N-benzoyl-DL-arginine-*p*-nitroanilide as substrates. Liberated amino acids were measured by ninhydrin method and *p*-nitroaniline was measured at 400 nm.

Fibrinolytic activity

Fibrinolytic activity of purified enzymes was determined by the method of Astrup and Melertz (1952).

Milk clotting activity

Milk clotting activity was measured by the method of Khan *et al.* (1979).

Sodium dodecyl sulphate (SDS) polyacrylamide gel electrophoresis (PAGE) was performed by the method of Weber and Osborn (1969) with 10 % acrylamide gel. Each gel tube (0·5 × 8 cm) was charged with 60–100 μg of protein and a constant current of 6 mA per gel tube was passed. Staining was performed with 1 % amido black in 7 % acetic acid. Excess stain was removed by 7 % acetic acid with frequent changes. After destaining, gels were stored in 2 % acetic acid.

Molecular weight determination

*Sephadex G-*100 *gel chromatography*: Molecular weights of purified proteases were determined by the method of Andrews (1964) by gel filtration on Sephadex G-100 using lysozyme (14,300), pepsin (34,700). β-lactoglobulin (18,400), trypsinogen (24,000), and ovalbumin (45,000) as standard protein. Samples of solutions containing nearly equal amount of standard proteins and purified proteases (1·0–1·5 mg/ml) in 0·02 M sodium phosphate buffer, pH 7·0 were loaded on previously equilibrated Sephadex G-100 column (1·6 × 50 cm) and eluted with same buffer containing 0·1 M NaCl. Fractions of 5 ml each were collected at a flow rate of 30 ml/h.

SDS-PAGE: Standard proteins and purified enzymes were incubated in 1 % SDS and 0·1 % β-mercaptoethanol in sample buffer for 2 h at 37°C. Rest of the method was as described earlier.

Kinetic studies: K_m and $V_{\max}$ were calculated from Lineweaver Burk plots prepared by using the method of least squares. Activation energies were calculated from Arrhenius plot.

Purification of proteases produced in the absence and in the presence of fructose

Purification steps used are indicated in table 1. Ammonium sulphate precipitate obtained at 60–90 % saturation was dissolved in a small amount of 0·02 M sodium phosphate buffer, pH 7·0 and was dialyzed overnight against the same buffer. Any sediment formed was removed by centrifugation and the supernatant was loaded on DEAE-cellulose column (31 × 1·6 cm) previously equilibrated with 0·02 M sodium phosphate buffer, pH 7·0. Column elution was performed by the same buffer with an increase in molarity from 0·02–0·5 M by inclusion of NaCl. Protease activity was assayed at pH 7·0 and the peaks obtained were in turn tested for optimum pH. Fractions showing maximum activity in the respective peak areas were pooled, concentrated, analyzed and then separately loaded on Sephadex G-75 columns (50 × 1·6 cm) and were eluted with 0·02 M sodium phosphate buffer, pH 7·0. A single peak of activity (measured at respective pH optimum) was obtained in each case and constituted the purified enzyme.

Proteases produced in the absence of fructose: As shown in figure 1, three peaks (I, II and III) obtained on DEAE cellulose chromatography corresponded to fractions 5, 23, and 35 respectively and had the optimum pH of 8·0, 9·5, 6·5, respectively and were designated as alkaline protease I (AP I), alkaline protease II (AP II) and neutral

Table 1. Purification of proteases produced by *A. alternata.*

Steps	Volume (ml)	Proteins (mg/ml)	Activity (U/ml)	Specific activity (U/mg protein)	Recovery (%)	Purification (fold)
Proteases produced in the absence of fructose						
Crude enzyme	200	3·64	1·91	0·52	100	1
Ammonium sulphate 60–90% ppt.	14	7·64	21·82	2·85	80·17	5·48
DEAE cellulose chromatography						
AP I	16	0·55	14·06	25·5	53·7	49·3
AP II	16	0·29	3·45	11·53	14·3	22·63
NP	26	0·22	9·06	41·1	64·3	79·00
Sephadex G-75 chromatography						
AP I	10	0·15	17·89	119·2	46·8	229
AP II	10	0·13	3·50	26·71	9·16	51·2
NP	16	0·11	11·00	100	46·1	192
Proteases produced in the presence of fructose						
Crude enzyme	200	7·63	10·9	1·42	100	1
Ammonium sulphate 60–90% ppt	15	8·27	131·0	15·42	90·1	11·2
DEAE cellulose chromatography						
FAP I	16	0·55	68·20	124·0	50	87·3
FAP II	16	0·45	47·12	104·70	34·60	73·7
FNP	16	0·23	51·80	225·20	38·02	158·6
Sephadex G-75 chromatography						
FAP I	10	0·14	76·3	545	35	383·8
FAP II	10	0·11	41·42	376·5	19	265
FNP	10	0·09	37·8	420·0	21	295·7

Proteases produced in the presence of fructose: As shown in figure 1, 3 peaks were also obtained after DEAE-cellulose chromatography but with a significant difference. The peaks in this case corresponded to fraction no. 4, 12 and 23. The pH optima of these peaks were 8·0, 7·5 and 9·5, respectively. Thus fraction no. 4 and 23 corresponded to peak I and II produced in the absence of fructose, but peak III produced in the absence of fructose was however eliminated when fructose was introduced in the culture medium and a new peak appeared at fraction 12 which had a neutral pH optimum. The two alkaline proteases in this case were designated as FAP I and FAP II and the neutral protease as FNP (table 1).

Properties of purified enzymes

Enzyme purity: SDS-PAGE gave a major band with minor impurities for all the 6

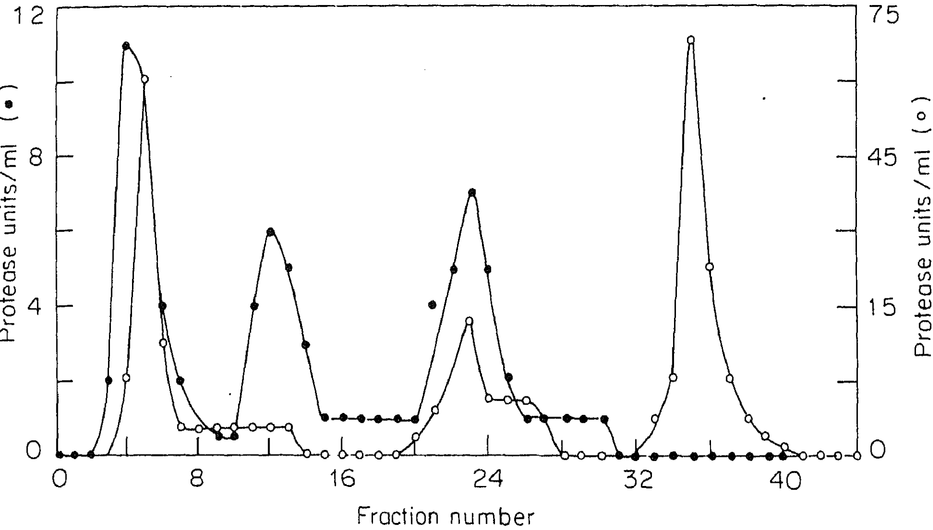


Figure 1. DEAE-cellulose chromatography of proteases of *A. alternata.* (O), Proteases produced in the absence of fructose; (●), proteases produced in the presence of fructose.

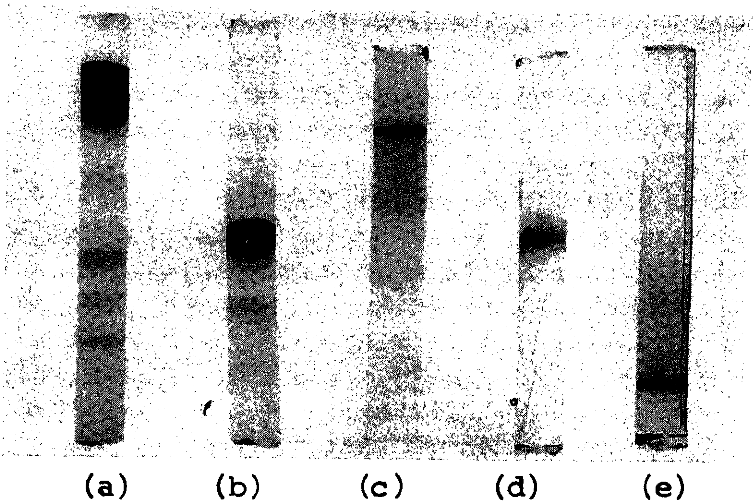
oH stability: Alkaline proteases and neutral proteases were stable over a pH of 7·0–10·0 and 6·0–8·0 when the enzymes were incubated for 2 h at 37°C in respective buffers.

Effect of temperature on activity and stability: AP I, AP II, NP, FAP I and FAP II exhibited maximum activity at 50°C but were stable upto 40°C in the absence of substrate whereas FNP showed maximum activity at 60°C and was stable in the absence of substrate at temperature upto 50°C when incubated for 20 min.

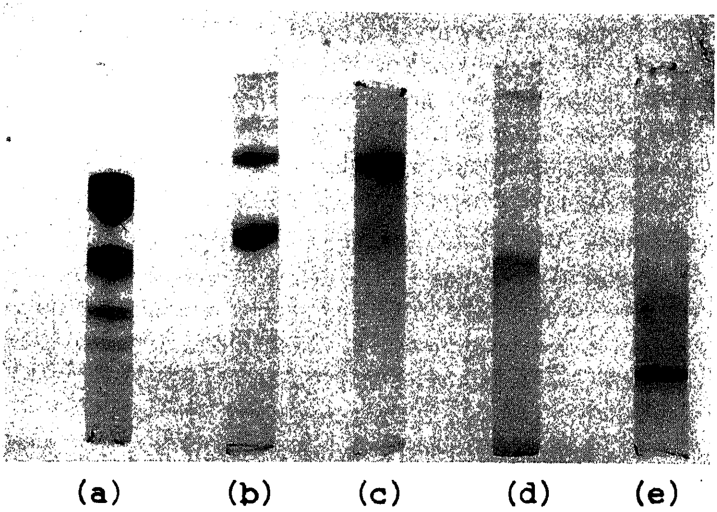
Activation energies: Ea of 5·39, 5·83 and 10·42 Kcal/mol were for AP I, AP II and NP respectively. These values for FAP I, FAP II and FNP were 3·5, 1·8 and 1·0 Kcal/mol respectively.

Kinetics: K_m values for AP I, AP II and NP were $4{\cdot}3 \times 10^{-4}$, $10{\cdot}5 \times 10^{-4}$ and $6{\cdot}3 \times 10^{-4}$ g/ml, respectively. The corresponding V_{max} were 198, 397, and 78 units/mg protein/h respectively. The K_m values for FAP I, FAP II and FNP were $5{\cdot}3 \times 10^{-4}$, $5{\cdot}2 \times 10^{-4}$ and $5{\cdot}3 \times 10^{-4}$ g/ml and V_{max} were 2640, 2100 and 1500 unit/mg protein/h, respectively.

A comparative account of various kinetic properties of the enzymes is given in table 2.



(2)



(3)

Figures 2 and 3. **2.** SDS-PAGE of proteases of *A. alternata* produced in the absence of fructose. (a), Culture filtrate; (b), 60–90% ammonium sulphate precipitate; (c), alkaline protease I (AP I); (d), alkaline protease II (AP II); (e), neutral protease (NP). **3.** SDS-PAGE of proteases of *A. alternata* produced in the presence of fructose. (a), Culture filtrate; (b), 60–90% ammonium sulphate precipitate; (c), alkaline protease I (FAP I); (d), alkaline protease II (FAP II); (e), neutral protease (FNP).

10^{-3}M and enzymes were incubated in buffer of appropriate pH for 10 min at 37°C prior to addition of substrate. All the above phenolic compounds tested were inhibitory of fructose.

Properties	AP I	AP II	NP	FAP I	FAP II	FNP
Fraction no. showing peak in DEAE-cellulose chromatography	5	23	35	4	12	23
Optimum pH	8·0	9·5	6·5	8·0	9·5	7·5
pH stability	7·0–10·0	7·0–10·0	6·0–8·0	7·0–10·0	7·0–10·0	6·0–8·0
Activation energy (Kcal/mol)	5·39	5·83	10·42	3·5	1·8	1·0
K_m (g/ml)	$4\cdot3 \times 10^{-4}$	$10\cdot5 \times 10^{-4}$	$6\cdot3 \times 10^{-4}$	$5\cdot3 \times 10^{-4}$	$5\cdot2 \times 10^{-4}$	$5\cdot3 \times 10^{-4}$
V_{max} units/mg protein/h	198	397	78	2640	2100	1500
Activation by L-cysteine-HCl (% activity)	108	110	164	100	100	100

Effect of inhibitors: As shown in table 3, metal chelating agents such as EDTA, *o*-phenanthroline, 8-OH quinoline had no effect. L-Cysteine-HCl stimulated NP but not FNP and alkaline proteases. Cysteine enzyme inhibitors such as β-mercaptoethanol, dithionitrobenzoic acid, *p*-chloromercuribenzoate, iodoacetate and TPCK inhibited both NP and FNP. Phenylmethylsulphonylfluoride (PMSF) a serine protease inhibitor strongly inhibited alkaline proteases.

Carboxypeptidase activity: None of the purified proteases had any carboxypeptidase activity.

Table 3. Effect of various inhibitors on activity of proteases of *A. alternata*.

	Activity(%)					
Compounds at 10^{-4}M	AP I	AP II	NP	FAP I	FAP II	FNP
None	100	100	100	100	100	100
L-Cysteine-HCl	108	110	164	100	100	100
EDTA	96·2	84·2	89·9	100	100	100
O-phenanthroline	95·2	85	90	85·2	80·0	95·0
8-OH Quinoline	96·2	78·2	90	100	100	95·0
β-Mercaptoethanol	96·2	78·2	—	85·2	80	10·5
PCMB	92·2	78·5	—	85·2	95·0	—
TPCK	92·2	60·0	—	87·2	95·0	—
Dithionitrobenzoic acid	96·2	60·0	5	87·2	80·0	—
Iodoacetate	95·0	60·0	—	85·0	79·0	—
PMSF	—	—	59·5	—	—	85·9

— Not detectable.

Above inhibitors at 10^{-4} M and enzymes in appropriate buffer of optimum pH were incubated for

Aminopeptidase activity: All the purified enzymes exhibited considerable aminopeptidase activity (table 4).

Fibrinolytic and milk clotting activity: All the purified enzymes had a fibrinolytic activity but none of the enzymes clotted buffered milk.

Esterase activity: A general characteristic of alkaline proteases is to catalyze hydrolysis of various esters and peptide bonds. AP I, AP II, FAP I and FAP II also hydrolyzed N-acetyl-L-tyrosine ethyl ester (NATE) indicating their ability to hydrolyse ester bonds. The optimum pH for NATE hydrolysis was 7·5 for AP I and FAP I, and for AP II and FAP II it was 8·5 with 0·2 M Tris-HCl buffer (data not given).

Action on various peptides and proteins: Hydrolytic action of purified proteases on various peptides and proteins is shown in table 5. It was seen that dipeptides,

Table 4. Aminopeptidase activity of proteases of *A. alternata*.

	Activity (units/ml)					
Substrate	AP I	AP II	NP	FAP I	FAP II	FNP
Leu-gly-gly	2·14	1·40	1·50	10·70	7·0	9·0
DL-Leu-gly-gly	2·57	1·65	1·75	12·84	10·80	11·5
Leu-*p*-nitroanilide	4·8	5·8	6·0	24·00	29·00	35·5
N-Benzoyl-DL-arginine-*p*-nitroanilide	3·0	3·6	4·8	21·00	16·64	24·1

Details as in text. One unit of aminopeptidase activity is expressed as liberation of 1 μmol of amino acid (leucine) or *p*-nitroaniline from the substrate per min under the assay conditions.

Table 5. Action of proteases of *A. alternata* on various peptides and proteins.

Peptide/protein	AP I (O.D.)	AP II (O.D.)	NP (O.D.)	FAP I (O.D.)	FAP II (O.D.)	FNP (O.D.)
Gly-leu	0	0	0	0	0	0
Gly-asp	0	0	0	0	0	0
L-ala-ph-ala	0	0	0	0	0	0
L-ala-leu	0	0	0	0	0	0
Ala-gly-gly	0	0	0	0	0	0
γ-gly-cys-gly	0	0	0	0	0	0
Leu-gly-ph-ala	0·07	0·05	0·10	0·11	0·105	0·07
Leu-gly-gly	0·15	0·10	0·12	0·19	0·18	0·12
DL-leu-gly-gly	0·15	0·11	0·09	0·20	0·19	0·09
N-CBZ-L-gly	0	0	0	0	0	0
N-CBZ-gly-ala	0	0	0	0	0	0
N-CBZ-L-leu-L-tyr	0	0	0	0	0	0
N-CBZ-gly-NH_2	0	0	0	0	0	0
N-CBZ-gly-ph-ala-NH_2	0	0	0	0	0	0
N-CBZ-OH-pro	0	0	0	0	0	0
L-ala-ph-ala-gly-gly	0	0	0	0	0	0

tripeptides and CBZ derivatives of various di- and tripeptides were not hydrolyzed by any of the proteases; but tripeptides possessing leucine at N-terminal end and proteins such as casein and bovine serum albumin were readily hydrolyzed.

Molecular weight determination: By Sephadex G-100 gel chromatography molecular weights of AP I, AP II and NP were 45,000, 30,500 and 18,500, respectively. Molecular weights of FAP I, FAP II and FNP were 45,000, 30,500 and 27,000, respectively.

SDS-PAGE: AP I, AP II, and NP exhibited molecular weights of 44,000, 30,250 and 18,000 and for FAP I, FAP II and FNP the values were 44,000, 30,250 and 28,000, respectively.

Discussion

Results presented in this paper indicate that extracellular proteases produced by *A. alternata* can be separated into two alkaline and one neutral protease. It appears from the data obtained that all the purified enzymes are predominantly endopeptidases as they hydrolyzed larger proteins such as casein and bovine serum albumin. They also hydrolyzed substrates such as leu-gly-gly, DL-leu-gly-gly, leu-*p*-nitroanilide and N-benzoyl-DL-arginine *p*-nitroanilide indicating that the enzymes hydrolyse peptides, amides and esters especially at the bonds involving leucine and arginine. In addition the alkaline proteases were serine proteases and the neutral proteases were cysteine proteases. As described in this paper, these enzymes can be purified to a considerable extent by using conventional technique.

A study of various kinetic and physicochemical properties indicates that although these properties are generally comparable to proteases produced by other fungi, certain important differences exist. Activation energies of these enzymes were found to be generally lower than those reported in the literature. An alkaline protease from *Penicillium caseicolum* has activation energy of 12 Kcal/mol (Khan *et al.*, 1979) and a protease from *Penicillium roqueforti* has 8 Kcal/mol of activation energy (Modler and Brummer, 1974). K_m values of *Alternaria* proteases tended to be higher than those of *Fusarium* (Modler and Brummer, 1974) which was $0{\cdot}5 \times 10^{-5}$ g/ml and lower than *Fusarium vasinfectum* which was 8×10^{-4} g/ml (Shrinivas *et al.*, 1967). However, AP II has a higher K_m value.

Stimulation of protease activity by Co^{2+} and Mn^{2+} has also been reported by many workers (Grippon and Hermer, 1974; Lysenkov, 1976). It was found that such a stimulation is generally higher for neutral proteases. Data on effect of phenolic compounds on protease activities is scanty. In our studies it was found that most of the phenolics except ferulic acid were inhibitory. Alkaline proteases of *A. alternata* exhibited both esterolytic and proteolytic activities. Similar to the observations made by us, several other proteases from microorganisms have been shown to possess fibrinolytic activity in addition to caseinolytic activity (Ushakova *et al.*, 1974; Egorov *et al.*, 1972).

from each other with respect to their behaviour in DEAE-cellulose chromatography, SDS-PAGE and cysteine activation. Fructose affects the protein patterns of the culture medium as indicated by SDS-PAGE presented in figures 2 and 3. Secondly, fructose also appears to enhance the production of AP I and AP II. Specific activities of these enzymes produced in the presence of fructose (*i.e.* FAP I and FAP II) were considerably higher than those produced in normal medium (*i.e.* AP I and AP II). It was also seen that the degree of purification obtained with FAP I and FAP II and FNP was also higher. Some kinetic properties of FAP I and FAP II such as activation energy and V_{max} were different from AP I and AP II, although there was no significant difference in K_m. This probably indicates modification of sites other than active sites. Thus, fructose might alter some properties of AP I and AP II and that this might be a contributory factor in the higher activities of proteases observed in a medium supplemented with fructose.

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Abstract. An increased tRNA methylase activity (100%) accompanied by a 40% decrease in the regulatory glycine methyltransferase activity was demonstrated in livers of mice fed on the carcinogenic (thioacetamide) diet, long before the onset of malignant transformation. Short-term treatment with thioacetamide and phenobarbital independently, also brought about a significant increase in the rat liver tRNA methylase activity. A significant increase in the tRNA methylase activity was observed in the mammary glands of pregnant as well as lactating mice as against the negligible enzyme activity in the normal mammary glands of C_3H and CBA mice, whereas a large increase in the tRNA methylase activity was evident in the spontaneously induced mammary tumours in these strains. Hepatic tRNA methylase activity was shown to remain unaffected in rats during various physiological stress conditions. It is suggested that elevation in the tRNA methylase activity may be one of the prerequisites during malignant transformation. A considerable increase in the tRNA methylase activity in host tissues of the tumour-bearing mice was also demonstrated.

Keywords. tRNA methylases; thioacetamide hepatocarcinogenesis; mouse mammary tumours; lactating mammary glands; pregnant mammary glands; host tissues; stress conditions.

Introduction

Alterations in tRNAs have been observed in a number of tumours (Borek and Kerr, 1972). Although modification of the structure of tRNA can be achieved in many ways, most common among these are the enzymatic insertion of methyl groups into the bases of the preformed macromolecule. Thus, marked changes in the activity of tRNA methylases in a variety of neoplasms have been reported (Borek and Kerr, 1972). Increased excretion of methylated purines has also been observed in tumour-bearing animals (Mandel *et al.*, 1966; Mcfarlane and Shaw, 1968). Several lines of evidence support the premise that there is no correlation between the rate of growth of tumours and the level of tRNA methylases (Borek and Kerr, 1972; Gantt and Evans, 1969; Pillinger and Wilkinson, 1971; Rodeh *et al.*, 1967). The high tRNA methylase activity in tumour tissues is also comparable to that in the foetal tissues. A number of similarities between foetal and tumour tissues have been observed such as foetal antigens (Masopust *et al.*, 1968) and novel isoaccepting tRNA species (Holland *et al.*, 1967; Yang, 1971).

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Abbreviations used: SAM; S-Adenosyl-L-methionine; TCA, trichloroacetic acid; GMT, glycine methyltransferase.

Although increased activity of tRNA methylases has been found in a very wide range of tumours, studies on tRNA methylases during premalignant or earlier stages are lacking. It has been hypothesized that aberrant methylation by tRNA methylases could lead to malignant transformation. In the present investigation an attempt has been made to study tRNA methylases in animal tissues during carcinogenesis, long before the onset of malignant transformation. Two systems were utilized for these studies, one that of chemically (thioacetamide) induced primary hepatocarcinogenesis and another that of spontaneously induced breast tumours in mice. These studies were also extended to the host tissues of tumour-bearing mice. The tRNA methylase activity was further investigated following injections of thioacetamide and phenobarbital as well as under various physiological stress conditions in rats.

Materials and methods

Chemicals

[Methyl-^{14}C] S-Adenosyl-L-Methionine (55 mCi/mmol) was obtained from the Radiochemical Centre, Amersham, England. Thioacetamide was obtained from E. Merck, Darmstadt, Germany. Hydrocortisone hemisuccinate and dioxane were purchased from Glaxo laboratories, Bombay. *Escherichia coli* B tRNA, phenobarbital (sodium salt), 2,5, diphenyloxazole, sucrose, Tris, glycine, adenosine, β-mercaptoethanol, and dithiothreitol were obtained from Sigma Chemical Co., St. Louis, Missouri, USA. Magnesium chloride and toluene were purchased from Sarabhai Chemicals, Baroda. Naphthalene and trichloroacetic acid were procured from British Drug House, Poole, England, and omnifluor from New England Nuclear, Pilot Chemicals Division, Boston, USA.

Treatment of animals

Mice of different strains were utilized for these studies: (i) Swiss mice for thioacetamide induced hepatomas, (ii) C_3H and CBA strains of mice for spontaneous mammary tumours and (iii) AKR mice for spontaneous leukemia.

A large group of Swiss mice, 6 to 8 weeks old, was fed with thioacetamide a non-alkylating hepatocarcinogen at a dose of 0·032 % in 20 % protein diet for 10–11 months. Under these conditions it takes about 15–17 months of continuous feeding of the carcinogen to induce frank hepatomas (Gothaskar *et al.*, 1970).

For short term studies with thioacetamide, 2 groups of Wistar rats, 10–12 weeks old, were injected each with the carcinogen (50 mg/kg body wt) daily for 3 days and (250 mg/kg body wt) daily for 8 days respectively.

Phenobarbital treatment—phenobarbitone (sodium salt) was made up in distilled water and injected intraperitoneally to Wistar rats in a daily dose of 100 mg/kg body wt for 3 days. It was also injected to Swiss mice in a daily dose of 75 mg/kg body wt for 6 days. The animals were sacrificed 24 h after the last dose.

The spontaneous breast tumours were normally observed in C_3H and CBA strains of mice after 4–5 pregnancies. Mammary tumours as well as mammary glands from pregnant as well as lactating mice, from the corresponding strains were taken for tRNA methylase studies which were also extended to the host livers of the tumour bearing mice. Another strain of mice namely AKR which is known to develop spontaneous leukemia after 7–8 months was used particularly for studying tRNA methylase activity in the host tissues such as liver and spleen.

Radiation effects

Rats (10–12 weeks old) were confined to a rectangular perspex box and exposed to X-rays from a Siemens Stablipan unit operated at 250 KV and 15 mA using 2 mμ aluminium filters. A dose rate of 100 r/min was obtained at a distance of 63·5 cms from the source where the animals were placed and the total dose delivered was 700 rads. The animals were sacrificed 24 h post irradiation.

Partial hepatectomy

Rats (10–12 weeks old) were hepatectomized under anaesthesia and about 70% liver was removed. The animals were maintained on saline for 24 h and on laboratory stock diet thereafter until the hour of sacrifice.

Adrenalectomy

Both male and female rats were adrenalectomized under anaesthesia and maintained on saline until they were sacrificed at 24 h and 48 h post operation.

Hormonal treatment

Hydrocortisone hemisuccinate (20 mg/kg body wt) was injected to a group of Wistar rats which were sacrificed 4 h later.

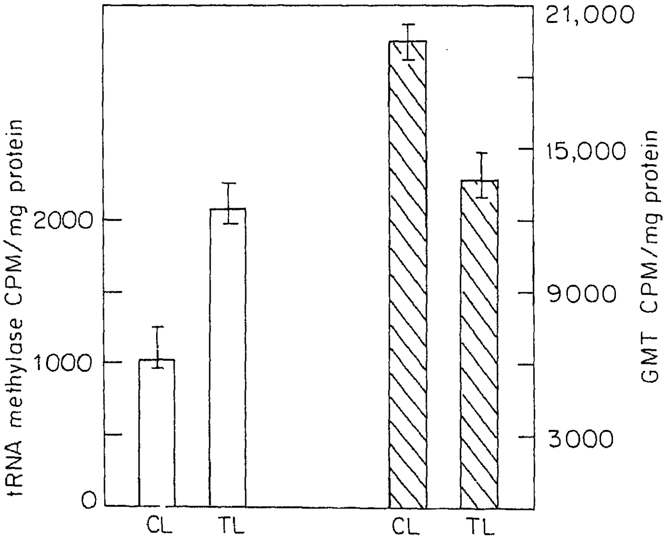
Enzyme assays

tRNA methylases: Animals were sacrificed by cervical dislocation. The post-microsomal supernatants obtained from tissue homogenates in 0·25 M sucrose containing 0·01 M $MgCl_2$ were used for determining the activity of tRNA methylases which was assayed by measuring the incorporation of [^{14}C]-methyl groups of S-adenosyl-methyl [^{14}C] methionine (SAM) into *E. coli* B tRNA. The system was essentially the same as described by Turner and Hancock (1971). The assay mixture in a final volume of 0·5 ml contained 50 μmol of Tris-HCl (pH 7·2), 5 μmol of $MgCl_2$, 5 μmol of β-mercaptoethanol, 100 μmol of *E. coli* B tRNA and 400 μg of supernatant protein. The reaction mixture was incubated at 37°C for 30 min followed by addition of 5 ml cold 5% trichloroacetic acid (TCA). After chilling in ice for 30 min the insoluble material was collected by filtration through millipore filter circles (pore size 0·45 μ) methylase activity was expressed as c.p.m/mg protein/30 min. Proteins were estimated by Lowry's method (Lowry *et al.*, 1951).

Glycinemethyltransferase (*GMT*): Assay of GMT was carried out by the method of Kerr (1972). The high speed pH 5 supernatant neutralized with 1 M Na_2CO_3 was used as a source of GMT. The reaction mixture contained 10 μmol Tris-HCl buffer (pH 8·2), 2 μmol dithiothreitol, 2 μmol glycine, 0·1 μCi (methyl [^{14}C] SAM and 400 μg enzyme protein in a final volume of 0·2 ml. The mixture was incubated at 37°C for 30 min. The reaction was terminated by adding 0·25 ml 20% phosphotungstic acid (PTA). Adenosine (1 μmol) was added to form a carrier precipitate. The volume was made to 2 ml and centrifuged for 30 min. Aliquots (0·3 ml) from the supernatant containing radioactive sarcosine were taken in cocktail D and counted in a liquid scintillation counter. The enzyme activity was expressed as c.p.m/mg protein/30 min.

Results and discussion

It can be seen that there is a 100% increase in the liver tRNA methylase activity at 10–11 months of feeding the thioacetamide diet as compared to the corresponding controls (figure 1). It may be emphasized that at this stage there was no histopathological evidence of malignant transformation which takes over 15 months feeding of the carcinogenic diet. It can also be seen that the GMT activity which is known to regulate liver tRNA methylase activity is decreased by about 40% at 10–11 months of the carcinogenic diet feeding, thus indicating a significant metabolic change long before the



Effects of carcinogen administration on hepatic tRNA methylase activity has been investigated earlier in animals. Hancock (1968) observed increased tRNA methylase activity in mouse liver after feeding 0·25% ethionine diet for 6 months. Such changes could also be observed in 10 days if 1% ethionine was given in the diet (Hancock, 1971). Hancock and Forrester (1973) have shown that 2 hepatocarcinogens, ethionine and dimethylaminoazobenzene (DAB) brought about an increase in the liver tRNA methylase activity after short term feeding of diet containing 1% carcinogen to rats, whereas 2 non-carcinogenic analogs methionine and norleucine had no effect on the tRNA methylase activity when similarly fed to rats.

In the present experiments, thioacetamide, a relatively weak hepatocarcinogen was administered in a 20% protein diet at a dose of only 0·032% with a view to assess the activity of tRNA methylases under conditions of hepatoma induction. When thioacetamide was injected (intraperitoneally) to rats in 2 doses of 50 mg and 250 mg per kg body wt for 3 days and 8 days, respectively, a significant increase in the tRNA methylase activity was observed, particularly at the latter dose (figure 2). However, even at such a high dose, the GMT activity was not significantly decreased, thereby indicating an immediate effect of thioacetamide other than the gradual metabolic change brought about by the carcinogen feeding. Thioacetamide, in short term experiments, is known

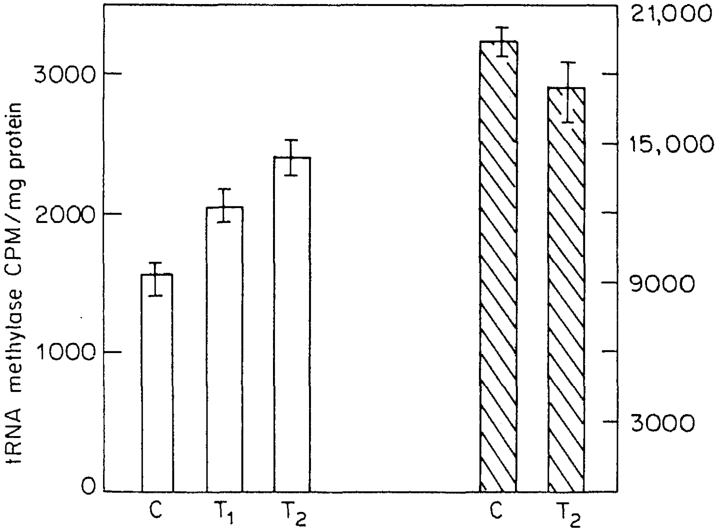
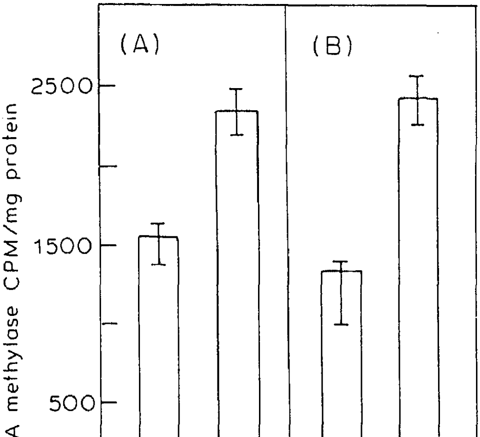


Figure 2. tRNA methylase and GMT activities in rat liver following short term treatment of thioacetamide. Blank bars: tRNA methylase; Solid bars: GMT. C, Control; T_1, thioacetamide injected-50 mg/kg body wt daily for 3 days; T_2, thioacetamide injected-250 mg/kg body wt daily for 8 days.

Each value is a mean of 4–5 independent experiments.

to cause nucleolar hypertrophy with a burst in the synthesis of rRNA (Villalobos *et al.*, 1964). It could be that the increase in the tRNA methylase activity is related to the nucleolar hypertrophy in short term experiments. Thioacetamide is known to be rapidly metabolised in the rat (Nygaard *et al.*, 1954) and hence, is relatively less toxic. In earlier work from this laboratory (Bhattacharjee *et al.*, 1972), nuclear hypertrophy has been observed after long term feeding of thioacetamide (0·032 % in 20 % protein diet) to rats. It may be that the observed increase in tRNA methylase activity in mice after 10–11 months feeding of the carcinogenic diet in the present experiments could be due to such a phenomenon. Increased tRNA methylase activity was also obtained after administration of phenobarbital to rats and mice in short term experiments (figure 3). Phenobarbital is a known promoter of hepatocarcinogenesis. It would be, therefore, of interest to evaluate the role of promoters on tRNA methylase activity in target tissues.

Results of tRNA methylase activity in spontaneously induced breast tumours in C_3H and CBA strains of mice are given in figure 4. These mammary gland tumours are known to occur spontaneously in older females after 4–5 pregnancies with an incidence of over 80 % in both the strains, whereas virgins are known to have a mammary tumour incidence of less than 20 %. It can be seen that breast tumours of both the strains of mice exhibit a high tRNA methylase activity as compared to that of mammary glands of the pregnant mice as well as that observed in the lactating mammary glands. It may be noted that the normal mammary gland has a negligible activity of tRNA methylases which is shown to increase during pregnancy and remain high during lactation. The competing enzyme GMT is absent in the normal mammary gland and has, therefore, no role in regulating the activity of tRNA methylases in this tissue.



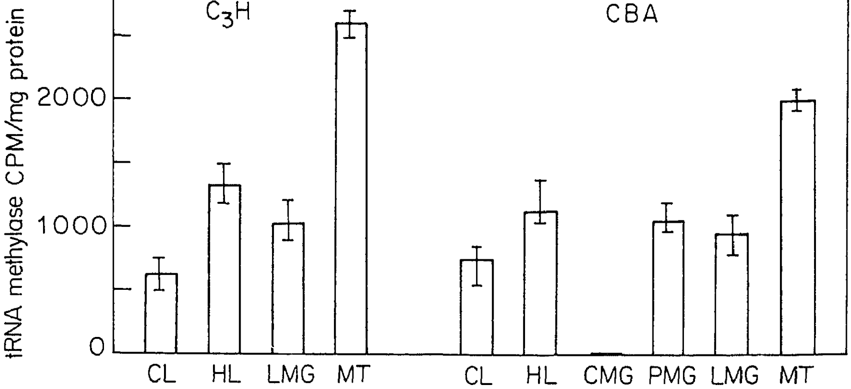


Figure 4. tRNA methylase activity in tissues of C_3H and CBA mice bearing spontaneous mammary tumours. CL, Control liver; HL, host liver; LMG, lactating mammary gland; MT, mammary tumour; CMG, control mammary gland and PMG, mammary gland of pregnant mice.

Each value is a mean of 4–5 independent experiments.

Elevation of tRNA methylases was observed by Turkington (1969) during the development of mammary gland in pregnant mice. These changes were attributed to differentiation in the epithelial cells of the mammary gland. Further, such an increase was also observed during mammary epithelial cell differentiation *in vitro* on addition of insulin, hydrocortisone and prolactin to the synthetic medium. Hormonal influence on tRNA methylases in target tissues has been well recognized (Pillinger *et al.*, 1971; Hacker, 1969; Sharma and Borek, 1970; Baliga and Borek, 1974). In the two susceptible strains that were used in the present studies it is possible that hormonal imbalance in the female breeders during pregnancy and lactation may ultimately give rise to the mammary tumours and that elevation of tRNA methylases may be one of the crucial steps leading to aberrant differentiation and malignant transformation/promotion.

The questions whether the tumour enzymes can lead to the formation of new methylated bases and whether such modifications affect the translation process have not been clearly understood (Turkington and Riddle, 1970; Brunke *et al.*, 1980; Leboy and Steiner, 1982).

Another interesting observation made in the present studies is increased tRNA methylase activity in host livers of the mammary tumor-bearing mice of C_3H and CBA strains (figure 4).

Figure 5 gives the results of tRNA methylase activity in host livers and spleens of mice of AKR strain bearing spontaneous leukemia. It can be seen that the enzyme activity is significantly increased in both these tissues. The spleens of these leukemic mice were enlarged about 20 fold in weight and were full of leukemic cells thus explaining the high level of tRNA methylase activity. In case of host liver, the high enzyme activity may be attributable to polyamine stimulation in addition to the possible infiltration of the leukemic cells. The increased levels of polyamines in rapidly

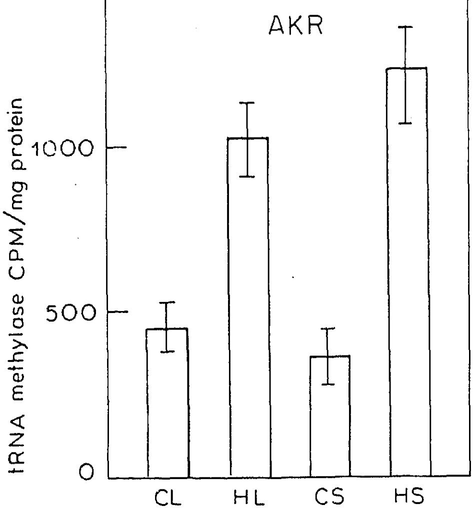


Figure 5. tRNA methylase activity in host tissues of AKR mice bearing spontaneous leukemia. CL, Control liver; HL, host liver; CS, control spleen and HS, host spleen.
Each value is a mean of 4–5 independent experiments.

reported (Russel and Snyder, 1968; Janne and Raina, 1968; Dykstra and Herbst, 1965; Russel and Lavy, 1971; Pegg, 1971). The differences between the high tRNA methylases of tumour tissues and the host tissue need further careful evaluation.

Results on the activity of tRNA methylases in rat liver following x-irradiation, partial hepatectomy, adrenalectomy and hydrocortisone treatment are summarised in table 1.

Table 1. Effect of physiological stress conditions on tRNA methylase activity in rat liver.

	tRNA methylase activity cpm per mg protein		
Treatment	4 h	24 h	48 h
Control	1150	1150	1150
X-irradiation (700 r)	—	1075*	—
Partial hepatectomy	—	1085*	1260*
Adrenalectomy	—	1070*	1015*
Hydrocortisone (2 mg/100 gm body wt)	1212*	—	—

Experimental details are given in the text. Control activity is the mean value of numerous experiments. Each value for different stress conditions is the mean of atleast four independent observations.

* Not significantly different from the control activity.

It can be seen that the tRNA methylase activity remains unaltered 24 h post irradiation (700 r) despite the fact that profound changes are known to occur in RNA and protein synthesis following x-irradiation (Pradhan and Srinivasan, 1971). Thus, it indicates that tRNA methylases cannot be easily subjected to alterations, the relative enzyme levels being remarkably constant. In case of *E. coli* B it is known that even if the organisms are exposed to ultraviolet radiation which is over 95% lethal, there is no change in the levels of tRNA methylases (Borek and Srinivasan, 1966).

It is evident from the same table that there is no change in the tRNA methylase activity at 24 and 48 h after partial hepatectomy as compared to that of controls. This would indicate, in the first instance, that changes in the tRNA methylase activity are not associated with a temporary stress condition and secondly, that the activity of these enzymes remains unchanged during normal rapid growth as assessed by *in vitro* methylation of heterologous substrate tRNA. These results are in agreement with those of Tsutsui *et al.* (1966) and Rodeh *et al.* (1967). However, Rodeh *et al.* (1967) observed high methylation of tRNA in regenerating rat liver in experiments *in vivo* which was attributable to increased synthesis of tRNAs after partial hepatectomy. Similar results under *in vivo* conditions were also obtained by Tidwell (1970). Stewart and Corrance (1969) did observe increased methylase activity in regenerating liver under *in vitro* conditions using ammonium ions and a purified preparation of the enzyme. According to these authors the increased levels of tRNA methylase activity obtained in 24 h regenerating liver, new born rat liver as well as those obtained in a minimal deviation hepatomas were only a reflection of the growth rate of a tissue. Such results however, have not been adequately substantiated.

Hydrocortisone administration (hormonal stress) to rats does not bring about any effect on the liver tRNA methylases as seen from the results (table 1). This is further borne out by the observed lack of change in the liver tRNA methylase activity following adrenalectomy (table 1). Although these results would rule out changes in the tRNA methylase activity on account of hormonal stress, these enzymes have been shown to be affected in non-target organs by very high doses of hormones (Shied and Wilson, 1970). It may be mentioned that hydrocortisone at a dose of 60 mg/kg body wt is known to induce *de novo* synthesis of tRNAs in rat liver (Wicks *et al.*, 1965).

It is evident from the foregoing results and discussion that tRNA methylases generally remain unaffected under various physiological stress conditions. The precise role of tRNA methylases and thereby, of specifically methylated tRNAs is yet to be elucidated. It would appear from the results of present studies that elevation of tRNA methylases due to lack of regulation, either on account of metabolic aberration or due to hormonal imbalance, is probably one of the prerequisites of malignant transformation. An intimate link between elevated tRNA methylases and malignancy has been provided by the fact that reversal or inhibition of oncogenicity is accompanied by diminution of the tRNA methylase activity (Fiume *et al.*, 1970; Halpern *et al.*, 1970). So far, only the methylases have been studied to a considerable extent and there may be several other modifying enzymes. There is, yet, a large lacuna in the understanding of normal regulatory mechanisms which may exist at the level of translation. Until subtle changes in the regulatory processes can be detected, the relationship between rapid

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Effects of protein deficiency on selective elicitation of lysosomal enzymes in rat peritoneal macrophages

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Abstract. Young albino rats were fed *ad libitum* 4, 8 or 20% (control) protein diet for 1–4 weeks. Total activities of some of the lysosomal enzymes, namely, acid phosphatase, aryl sulphatase, β-glucuronidase and cathepsin D, were determined in resident and protease-peptone elicited peritoneal macrophages. Total cell number, protein content and the lysosomal enzyme activities were increased significantly in protease-peptone elicited macrophages; though at a lower rate in 4% protein-fed group compared to control ones. However, the rate of induction of the tested hydrolases was selective and their response to the stimulant varied widely. Similarly, response of each enzyme to low protein diet also varied. Thus, at 4 weeks, cathepsin D and β-glucuronidase activities, expressed per total number of elicited macrophages were reduced by 45 and 60%, respectively, in 4% protein-fed animals. These results indicate that the metabolic events related to lysosomal function in macrophages, are affected by dietary restriction of proteins.

Keywords. Rat; protein deficiency; peritoneal macrophages; elicitation; lysosomal enzymes.

Introduction

The macrophage is an integral component of the immune system and is involved in a number of immune functions, besides in phagocytosis and cell-killing (Bursuker and Goldman, 1983). Nutritional status of an animal is closely related to its resistance to infection (Chandra, 1980) due to a variety of factors. Protein malnutrition results in reduction in relative number of elicited peritoneal macrophages in response to proteose-peptone stimulation (Iyengar and Vakil, 1985). Similarly, IgG-FC receptor mediated phagocytic capability (Hamm and Winick, 1984) and bactericidal activity (Douglas and Schopfer, 1974) of the macrophages are reduced in protein deficiency. Macrophages also contain large number of lysosomal enzymes (Davies *et al.*, 1974) which are shown to control many immunological activities of the cells (Unanue, 1978). The enzymes play specific role in intracellular digestion and in destructive inflammatory processes (Goodrum and Spitznagel, 1982). It is shown that the mobility of macrophages to inflammatory sites is depressed in protein deficiency (Weeks and Kavas, 1979).

In the present investigation, the effects of protein malnutrition on the activities of some selective lysosomal enzymes of resident and elicited rat peritoneal macrophages, were studied.

Materials and methods

Chemicals

4-Methylumbelliferone and its conjugated substrates, phenolphthalein glucuronide and bovine haemoglobin were procured from Sigma Chemical Co., St. Louis, Missouri, USA. Eagles' minimal essential medium and proteose-peptone were Difco products and purchased from HIMEDIA, Bombay. Potassium [^{14}C]-cynate (K^{14}CNO, sp. activity 57 mCi/m mol) was obtained from Amersham, England. Other chemicals were of analar grade.

[^{14}C]-haemoglobin preparation

An aqueous solution of haemoglobin (400 mg/20 ml, pH 6·1) was incubated with 0·4 ml of 0·04 mCi of K^{14}CNO for 2 h at 50°C (Peters *et al.*, 1972). The solution was kept for 16 h at 25°C and then incubated with 20 μmol of cysteine HCl (pH 6·1) for 2 h at 37°C. This was dialysed extensively against distilled water and 0·01 M Tris buffer (pH 7·4), till no radioactivity was detected in the washings. Specific activity of [^{14}C]-haemoglobin preparation was about 79,000 cpm/mg protein.

Animals

Young male albino rats of Wistar strain (about 100 g body wt) fed on animal house stock diet, (cereal-legume based diet containing 20% protein) were used. A number of animals were sacrificed before initiation of different dietary protein regimen to obtain the base line values for the parameters studied. Rest of the animals were randomly divided into three groups and fed *ad libitum* 4, 8 or 20% casein diet for 1–4 weeks. They were housed individually in cages at room temperature (25 ± 3°C). The composition of the diet (g per 100 g diet) was: corn starch (57), casein (20), sesame oil (8), sucrose (9), vitaminized sucrose (2) and salt mixture (4) (Adhikari *et al.*, 1972). In 8 and 4% protein diets, casein was replaced by equivalent amounts of starch. Daily food intake and body wt of the animals were recorded.

Collection of macrophages

For the collection of resident macrophages, the animal was injected intraperitoneally with 20 ml of 0·1 M phosphate buffer saline (PBS, pH 7·2) solution, massaged lightly and killed under ether anesthesia. The peritoneal fluid was collected, centrifuged (600 *g*, 10 min) and the cell pellet suspended into Eagles' minimal medium containing 100 μg/ml each of streptomycin and penicillin. This was transferred asceptically into light microscope and counted. They were identified (95%) as medium to large mononuclear cells. Viability (about 90%) of the cells was determined by exclusion of trypan blue.

Elicited exudate cells were collected as above from rat peritoneum, three days after intraperitoneal injection of 10% proteose-peptone (5 ml). Nonionic detergent triton X-100 (1%) in 0·9% NaCl was added to the cells and kept at 4°C for 1 h. Appropriate volume of the cell-lysates were stored at −20°C until analysed.

Biochemical determinations of lysosomal enzymes

Acid phosphatase (EC 3.1.3.2) and aryl sulphatase (EC 3.1.6.1) were assayed by fluorimetric method, using 4-methylumbilliferone (4-MU)-conjugated derivatives, namely, 4-MU-phosphate (0·4 mM) and 4-MU sulphate (10 mM), respectively, as the substrates (Mead *et al.*, 1955). An aliquot of the macrophage lysate (0·10 ml) was incubated with appropriate substrate (in acetate buffer, pH 5·0) at 37°C for 90 min. The reaction was stopped by addition of 2·5 ml glycine buffer (0·4 M, pH 10·5). The 4-MU liberated was determined fluorometrically in a Hitachi 203 fluorescence spectrophotometer at an excitation and emission wavelengths of 365 and 450 nm, respectively. A standard curve was drawn using known concentrations of 4-MU. The enzyme activity was expressed as nmol 4-MU liberated per min per 10^6 cells.

To measure β-glucuronidase (EC 3.2.1.31) activity (Stahl and Touster, 1971), lysed cell suspension ($\equiv$ approx. $1{\cdot}5 \times 10^6$ cells) was incubated with 0·625 mmol phenolphthalein β-glucuronide in 0·1 M acetate buffer (pH 5·0) at 37°C for 60 min. The reaction was terminated by the addition of 4 ml of a solution (pH 10·7) containing 0·133 M glycine/0·067 M NaCl/0·083 M Na_2CO_3. The colour intensity was measured at 550 nm. One unit of the enzyme was defined as one nmol of phenolphthalein liberated per min per 10^6 cells.

For the measurement of cathepsin D (EC 3.4.4.23) activity (Roth and Locty, 1971), [^{14}C]-haemoglobin (about 100,000 counts), 1·4 ml of 0·2 M acetate buffer (pH 3·8), and 1 ml of cell lysate, were incubated at 37°C for 90 min. The reaction was stopped by the addition of 5% trichloro acetic acid and then centrifuged. An aliquot of the supernatant was added to 10 ml of dioxane containing 10% naphthalene and 0·5% 2,5-diphenyl oxazole as scintillation fluid. Radioactivity was counted in a Beckman liquid scintillation counter. Corrections were made for quenching and background counts. The enzyme activity was expressed as cpm liberated per min per 10^6 cells.

Total proteins in macrophage lysate were determined (Miller, 1959) using bovine serum albumin as standard. The results were statistically analysed using Student's '*t*' test (Snedecor and Cochran, 1967). Differences in mean values yielding $P \leqslant 0{\cdot}05$ were considered significant.

Results

Growth

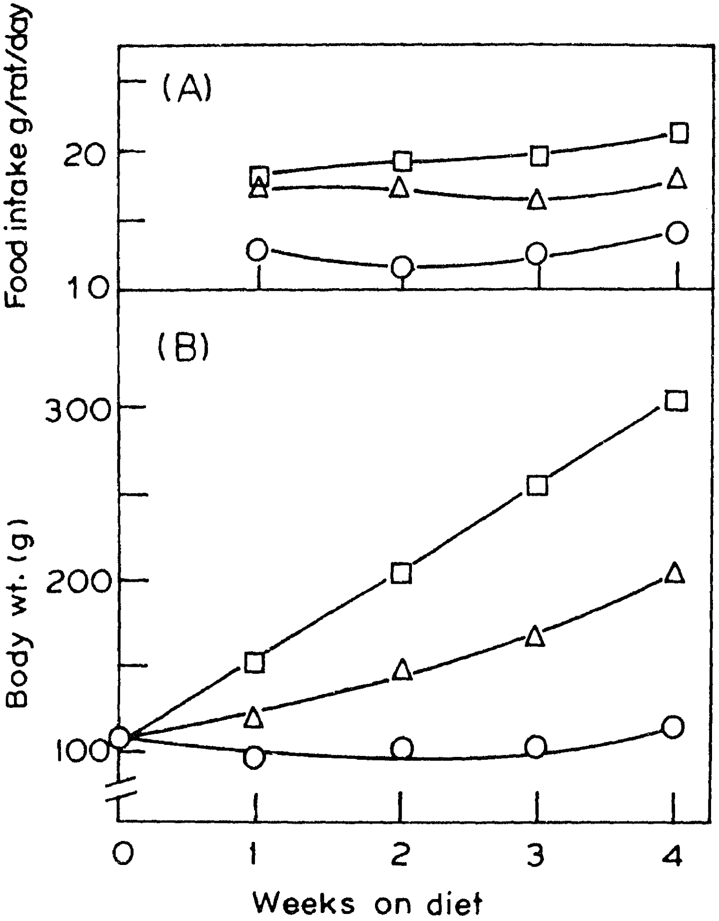

Figure 1. Food intake (g) and body wt gain (g) by rats, fed different levels of dietary proteins. Young male rats were fed 4 % (O) or 8 % (Δ) or 20 % (□) protein diet. Food intake and weight gain were recorded and plotted against number of weeks of feeding. Each point represents an average value for 8 rats.

was higher than that by the 4 % group (figure 1A). Animals fed adequate (20 %) and marginal (8 %) protein diets gained weight throughout the experimental period, though at a lower rate in the latter group (figure 1B). Animals fed inadequate (4 %) protein diet, did not gain weight but maintained essentially the initial level during 4 weeks.

Effects of protein deficiency on rat peritoneal macrophages

In the preliminary experiments, time course studies were carried out (figure 2) to determine the maximum response of the animals (fed 20, 8 or 4 % protein diets for 4

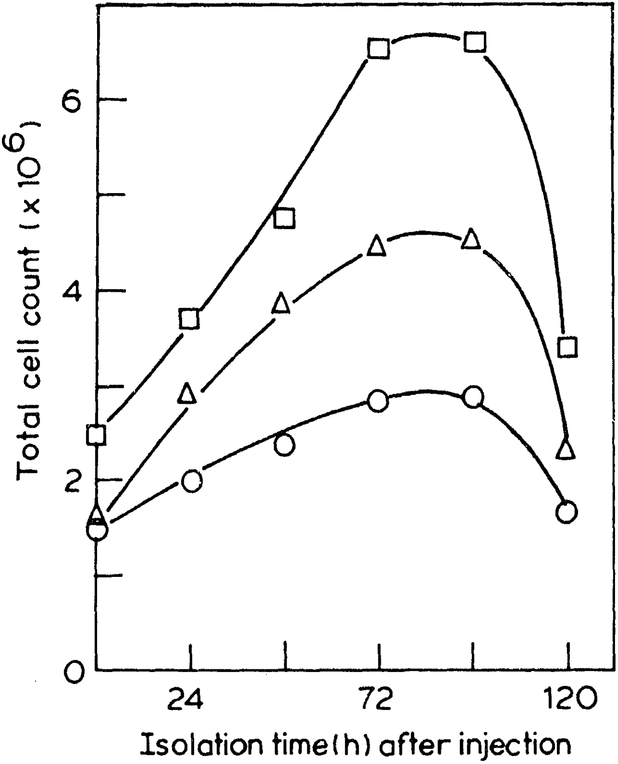

Figure 2. Total number of elicited macrophages collected at different time intervals following the administration of stimulant. Animals, fed for 4 weeks on experimental diets, were injected proteose-peptone and macrophages were collected after 24–120 h, as described in the text. (O), 4% protein; (Δ), 8% protein; (□), 20% protein.

can be observed that the pattern of exudative response to the stimulant was similar in all the groups, though the rate of increase in the number of cells was very slow in protein-deficient group compared to the control. Hence in all subsequent experiments, the animals were sacrificed at 72 h.

The results on the total number of elicited peritoneal macrophages, recovered from the animals fed different dietary protein levels for 1–4 weeks, are shown in table 1. In 20% protein fed group, the total number of macrophages were $4{\cdot}64 \times 10^6$ cells. This value was comparable to the basal control value ($4{\cdot}5 \times 10^6$ cells) on 0 day, obtained from the animals, fed stock diet, before initiation of dietary protein regimen. However, the value for 8% ($3{\cdot}88 \times 10^6$) and 4% ($3{\cdot}58 \times 10^6$) protein fed groups were slightly lowered during this period. Further, total population of elicited macrophages increased steadily in the control group, whereas it was reduced markedly ($P < 0{\cdot}05$) in the protein-deficient (4%) animals at 4 weeks compared to that in 20% protein-fed animals. Thus,

Table 1. Effects of dietary protein levels on total elicited peritoneal macrophages.

Week on diet	Dietary protein level (%)					
	20	8	4	20	8	4
	(Total cells × 10^6)			(μg protein/10^6 cells)		
0	4·50 ± 0·50			210·3 ± 5·46		
1	4·64 ± 0·83	3·88 ± 1·07	3·58 ± 0·30	197·5 ± 46·91	184·30 ± 19·24	174·0 ± 53
2	7·68 ± 1·04	4·92 ± 0·29	3·20 ± 0·41	227·0 ± 25·70	147·30 ± 27·10	141·6 ± 9·
4	8·32 ± 1·56	3·97 ± 0·74*	2·50 ± 0·55*	200·8 ± 19·62	145·15 ± 22·60*	138·7 ± 17

Macrophages were separated from peritoneal exudate, 72 h after the injection of the stimulant. Each va the average of 8 individual determination ± S.E.

* $P < 0·05$

control group during the experimental period of 4 weeks. However, this was redu significantly ($P < 0·05$) in the 4% protein fed group compared to control.

Protein status and macrophage lysosomal enzymes

In preliminary experiments, optimum assay conditions for each macrophage enz studied, namely, acid phosphatase, aryl sulphatase, β-glucuronidase and cathepsi were established individually. Time course and kinetic studies showed (data not gi that all the enzyme assays were linear with time, though having different peak activ (up to 3 h). These were also proportional to the amount of enzyme concentra (sample size up to 2 × 10^6 cells) at 37°C and at their specific pH optimum (3·8 cathepsin D and 5·0 for other enzymes). These enzymes exhibit latent activity and released from the lysosomes by incubation with triton X-100.

Effect of protein deficiency on lysosomal enzyme activities

Data on the effects of protein status of an animal on the activities of acid phospha aryl sulphatase (table 2), β-glucuronidase and cathepsin D (table 3) in resident elicited macrophages are compiled. Data at 0 time represent the basal control va obtained from the animals, fed stock diet before initiation of protein dietary regir The activities of the four lysosomal enzymes (units/10^6 cells) in resident macroph were comparable to the initial (0 time) values and did not change significantly (P < in the 20 and 8% protein fed groups in 4 weeks, except, aryl sulphatase activity w showed an increase ($P < 0·01$). Though at 4 weeks, acid phosphatase and aryl sulpha activities were reduced significantly ($P < 0·02$) in the 4% protein-fed group compare that in control, cathepsin D activity showed some increase during this period.

The total lysosomal enzyme activities (units/10^6 cells) were increased mark in elicited macrophages in all the groups. However, at 4 weeks, acid phospha aryl sulphatase (table 2) and β-glucuronidase (table 3) activities were significa able 2. Effects of protein deficiency on lysosomal enzyme activities of resident and elicited rat peritoneal macrophages.

nzyme	Week on diet	Dietary protein level (%) 20 Resident	20 Elicited	8 Resident	8 Elicited	4 Resident	4 Elicited
		(units/10^6 cells)					
cid phosphatase							
	0	7·16 ± 0·90	31·43 ± 2·45				
	1	5·91 ± 0·86	27·32 ± 4·64	5·24 ± 0·36	20·67 ± 1·61	6·41 ± 0·65	30·24 ± 9·91
	3	6·16 ± 0·72	30·05 ± 1·97	5·55 ± 0·66	12·62 ± 2·37	4·98 ± 0·47	13·93 ± 1·43
	4	7·43 ± 0·77	29·26 ± 3·32	6·17 ± 0·83	17·82 ± 3·69	4·84 ± 0·44^a	17·94 ± 1·77^b
yl sulphatase							
	0	10·75 ± 0·40	22·10 ± 2·86				
	1	9·20 ± 1·30	22·71 ± 2·71	8·77 ± 2·18	24·06 ± 5·66	7·38 ± 1.40	23·70 ± 1·24
	3	11·10 ± 0·76	24·79 ± 2·61	7·11 ± 0·93	18·63 ± 4·51	9·60 ± 0·39	19·85 ± 1·58
	4	13·20 ± 1·47	26·05 ± 2·90	13·18 ± 1·46	20·26 ± 3·58	8·80 ± 0·78^a	18·76 ± 1·18^b

sosomal enzyme activities were measured in the resident and elicited macrophages as described in 'Methods'. 1 unit of acid phosphatase and aryl phatase activity relates to nmol of 4-MU liberated/min/10^6 cells. Each value is the average of 8 determinations ± S.E.
<0·02; ^{b} P <0·01.

Effects of dietary protein deficiency on lysosomal enzymes in resident and elicited rat peritoneal macrophages.

		Dietary protein level (%)					
	Week on diet	20		8		4	
		Resident	Elicited	Resident	Elicited	Resident	Elicited
nidase		(units/10^6 cells)					
	0	1·85 ± 0·12	3·35 ± 0·15				
	1	1·72 ± 0·32	2·91 ± 0·06	1·52 ± 0·19	2·44 ± 0·35	1·32 ± 0·10	3·64 ± 0·83
	3	1·61 ± 0·07	3·61 ± 0·41	1·31 ± 0·18	2·23 ± 0·51	1·34 ± 0·22	2·41 ± 0·41
	4	1·51 ± 0·13	3·19 ± 0·58	1·64 ± 0·13	2·61 ± 0·20	1·40 ± 0·18	2·26 ± 0·15[a]
D							
	0	78·91 ± 4·64	244·4 ± 29·41				
	1	69·95 ± 12·90	297·3 ± 56·85	72·93 ± 6·10	342·8 ± 24·24	73·25 ± 9·31	339·7 ± 83·60
	3	82·45 ± 13·80	271·8 ± 23·74	86·50 ± 7·50	308·4 ± 52·70	73·98 ± 10·72	280·9 ± 32·82
	4	77·70 ± 14·40	264·6 ± 42·30	84·50 ± 12·70	284·0 ± 38·70	94·35 ± 14·91	263·3 ± 41·30

enzyme activities were measured in the resident and 72 h elicited macrophages as described in 'Methods'. 1 unit of β-
ase activity is equivalent to nmol of phenolphthalein liberated/min/10^6 cells. One unit of cathepsin D activity relates to
ctivity in supernatant liberated from labelled haemoglobin (cpm/min/10^6 cells). Each value is the average of 8
ions ± S.E.

examined. Varied and selective elevation of individual lysosomal enzymes in elicited macrophages was observed. Thus, at 4 weeks, cathepsin D showed 3·5 fold increase in total activity in proteose-peptone elicited cells compared to the resident ones in 20% protein-fed group. Whereas for β-glucuronidase, the stimulation effect was of low significance with only 2-fold increase under similar conditions. Changes in 4% protein-fed groups were similar, though the increment in the enzyme activities were slightly lower. Thus, cathepsin D and β-glucuronidase registered 2·8 and 1·6-fold increase, respectively, in elicited cells at 4 weeks. The pattern was more or less the same and not statistically ($P < 0{\cdot}1$) different during the 4 weeks in all the groups.

To have a better insight into the action of the stimulant, absolute increase in total enzyme units (per 10^6 cells) in elicited macrophages over that of resident ones was calculated (figure 3). At first week, increase in the number of enzyme units were comparable, or slightly higher in the 4% protein-fed group. However, at 4 weeks, all the enzymes were adversely affected, and their response to stimulation (in terms of total units/10^6 cells) was reduced.

Since the total number of elicited cells were reduced with progressive protein deficiency (table 1), the decrease in lysosomal enzyme activities in total peritoneal macrophages was more pronounced than when expressed per 10^6 cells. When the time related changes were examined, the decrease ranged from a minimum of about 45% in cathepsin D and β-glucuronidase activities to a maximum of about 60% in acid

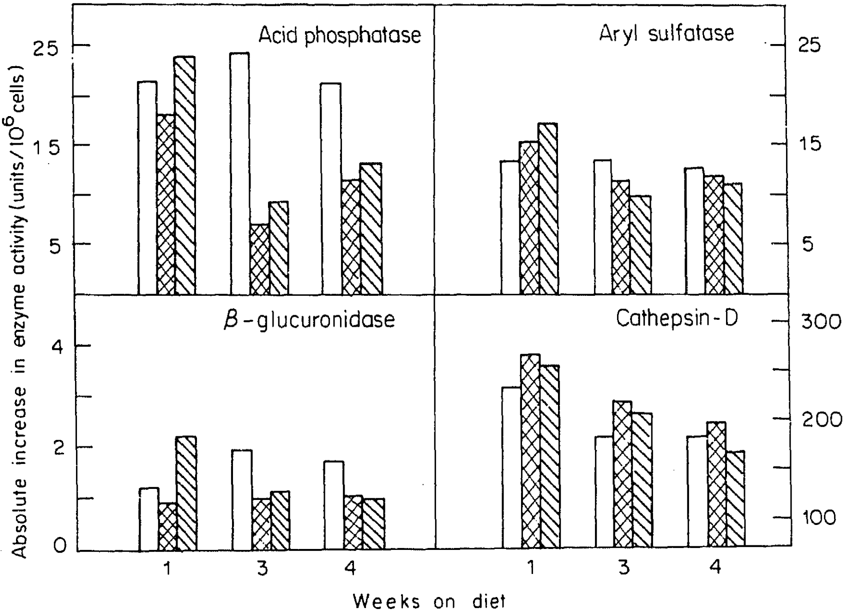

Figure 3. Absolute increase in the lysosomal enzyme activities in elicited macrophages. Three sets of bars indicate the time in weeks, for which the animals were fed 4% (☐), 8% (▩)

phosphatase activity from the first to the fourth week on protein-deficient diet. The total enzyme activities in 20% protein group increased from 60% (cathepsin D) to about 100% (aryl sulphatase) during the same period.

Discussion

The observations in the present study indicate that reduction in food intake in protein-deficient animals, produced a combined protein-calorie malnutrition. The growth of the animal was almost arrested due to inefficient utilization of low protein diet (figure 1).

As the severity of dietary protein malnutrition increased, the number of resident and elicited macrophages decreased proportionately. It is known that stimulated rat peritoneal macrophages synthesize lysosomal enzymes at higher rates (Brown and Swank, 1983). Recent evidences suggest that both, a mannose-6-phosphate recognition marker on most of the lysosomal enzymes (*e.g.* on β-glucuronidase, cathepsin D, etc.) (Hasilik and Neufeld, 1980) and phosphomannosyl receptor, an integral membrane glycoprotein (Creak and Sly, 1983) play a significant role in the intracellular transport of the newly synthesized enzymes from microsomal organelles to lysosomes. Overall decrease in total sugars, including mannose, has been observed in protein deficient macrophages (Iyengar and Vakil, 1985). Thus, low activities of the lysosomal enzymes in macrophages collected from protein-deficient rat at 4 weeks, may be attributed to (a) overall diminution in cell protein synthesis (table 1) and/or (b) impaired shuttling of newly synthesized lysosomal precursors from the site of synthesis to the lysosomes in mature form. Similar decrease in lysosomal enzymes in spleen and thymus was observed in protein deficient animals (Munoz *et al.*, 1981). These enzymes are shown to be involved in restructuring of lymphoid cells during their transformation in the presence of antigen (Hirschhorn and Hirschhorn, 1965).

However, it was noticed that cathepsin D activity (expressed per 10^6 cells) in elicited macrophages was comparable in both the groups (table 3). The significance of this observation is not clear. It can be speculated that comparatively higher proteolytic activity per cell, in protein deficient rat might be the expression of the mechanisms responsible for excess protein breakdown. This phenomenon may be stimulated by lack of appropriate amount of amino acids in the diet. Proteolytic enzymes in rat liver are shown to be involved in protein catabolism during malnutrition (Umana, 1967).

On scrutinizing the results more closely, it could be observed that proteose-peptone injection caused selective and differential stimulation of the enzymes studied. Interaction of stimulant with the intact cells and its subsequent endocytosis, are required for the induction of the lysosomal enzymes in macrophages (Tomino, 1979). The stimulant used being protein in nature, it could induce proteolytic enzymes like cathepsin D much more (3·5 fold) compared to β-glucuronidase and aryl sulphatase (about 2-fold) in control. Similarly, incubation of macrophages with liposomes is shown to induce β-glucuronidase but not β-glucosidase in lysosomes (Takano *et al.*, Many proteases, including cathepsin D augment immune response *in vitro* and *in vivo* and play important role in the regulation of phagocytosis by macrophages and in antibody production (Ohnishi, 1984). Malnourished subjects are more susceptible to infection than normal healthy one, since many aspects of immunocompetence are affected (Chandra and Newberne, 1977). Humbor *et al.* (1983) have shown that the migratory ability of the peritoneal macrophages is decreased in protein deficiency. Thus, reduced availability of macrophages, and consequently of the lysosomal enzymes at inflammation sites, may be one of a variety of factors, affecting the host–parasite interaction in protein deficiency.

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Changes in the subcellular distribution of brain and heart hexokinase isoenzymes during alloxan diabetes

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Abstract. Changes in the subcellular distribution of hexokinase activity from three brain regions and heart were studied during alloxan induced diabetes. There was an overall decrease in the particulate hexokinase with an increase in the soluble form, after different time intervals of the onset of diabetes. Administration of insulin to the diabetic rats showed a partial counteraction of the enzyme changes. A possible regulation of brain hexokinase by metabolite changes is proposed.

Keywords. Hexokinase; isoenzymes; brain; insulin; diabetes.

Introduction

Adult brain is dependent on glucose as the major substrate for energy metabolism (McIlwain and Bachelard, 1971). The key position of hexokinase (ATP: D-hexose 6-phosphotransferase, EC 2.7.1.1) in the cellular metabolism of glucose makes the study of this enzyme of great interest. Hexokinase in the brain is predominantly localized in the particulate fraction (Crane and Sols, 1953; Wilson, 1968) and, *in vivo* particulate and soluble forms of the enzyme remain in a state of dynamic equilibrium, contributing to the regulation of glycolysis in the central nervous system (Knull *et al.*, 1973).

The present results demonstrate the changes in the amount and subcellular distribution of the two isoenzymic forms of hexokinase from different regions of the rat brain under conditions of short and long term diabetes. Since effects of diabetes on heart tissue are well documented, heart tissue was also included in the study for comparison.

Materials and methods

Animals

Albino rats of Wistar strain, weighing between 200 and 300 gm were used for all the experiments. The rats were made diabetic with alloxan and were divided into two groups (8–12 rats were used in each group for the different time intervals), as described earlier (Murthy and Baquer, 1981). The first group of rats formed the diabetic group. To the second group, protamine zinc insulin was injected each day (2 units/day) until the day of sacrifice and these were designated as diabetic rats treated with insulin. Both groups were given food and water *ad libitum*.

Preparation of homogenates

Rats were killed by cervical dislocation and cerebral hemispheres, cerebellum, brain stem and heart were excised immediately and weighed. Tissue homogenates (1:10) were prepared as described earlier (Murthy and Baquer, 1981). The extracts were centrifuged at 12,000 *g* for 40 min. The 12,000 *g* supernatant was used as the soluble fraction. The pellet was washed once and then resuspended in the homogenizing medium, and formed the total particulate fraction (TPF).

Enzyme assay

Hexokinase was estimated essentially according to the method of Sharma *et al.* (1963) as modified by Gumaa and McLean (1972). The TPF was treated with Triton-X-100 (final concentration 0·5 %) in the cold for 30–60 min to liberate the latent and bound enzymes. For the estimation of Type II hexokinase activity, aliquots of each fraction were heated at 45°C for 1 h (Grossbard and Schimke, 1966). This process destroyed hexokinase Type II completely. A unit of hexokinase activity was defined as the amount required to form 1 μmol of NADPH per min at 25°C.

Blood glucose was estimated according to the method of Bergmeyer *et al.* (1974).

ATP, glucose-6-phosphate dehydrogenase, NADP and Tris were obtained from Sigma Chemical Co., St Louis, Mossouri, USA. All the other chemicals were from British Drug House, UK and were of analar grade.

Results

The severity of diabetes was established by the determination of blood glucose levels, which increased significantly from control values of 5·5 ± 0·3 mM to 26·5 ± 2·3, 28·8 ± 0·9 and 31·6 ± 1·2 mM at 3, 8 and 15 days, respectively after induction of diabetes. Administration of insulin to diabetic rats (D + I group) decreased the blood glucose level to 15·4 ± 0·8, 14·6 ± 1·3 and 12·9 ± 0·9 mM on 3, 8 and 15 days, respectively.

Changes in hexokinase activity in brain regions

Table 1. Effect of alloxan diabetes and insulin administration on total particulate hexokinase from brain and heart.

	Cerebral hemispheres		Cerebellum		Brain stem		Heart	
Time (days)	D	D + I	D	D + I (units/gm)	D	D + I	D	D + I
3	$7{\cdot}4^{c} \pm 0{\cdot}4$	8·6 ± 0·4	5·6 ± 0·3	6·1 ± 0·3	3·1 ± 0·2	2·7 ± 0·2	$0{\cdot}78^{c} \pm 0{\cdot}07$	$1{\cdot}2^{b} \pm 0{\cdot}1$
8	$7{\cdot}3^{c} \pm 0{\cdot}3$	9·7 ± 0·6	5·3 ± 0·2	5·3 ± 0.02	2·8 ± 0·2	3·2 ± 0·2	$1{\cdot}3^{a} \pm 0{\cdot}08$	1·8 ± 0·03
15	$7{\cdot}5^{c} \pm 0{\cdot}1$	9·3 ± 0·4	5·8 ± 0·2	5·9 ± 0·4	3·1 ± 0·2	3·4 ± 0·04	1·3 ± 0·1	1·6 ± 0·04
Control	9·1 ± 0·2		5·9 ± 0·2		3·1 ± 0·2		1·6 ± 0·1	

Each value is a mean ± S.E.M. of four or more experiments done in triplicates.
D = Diabetes and D + I = diabetes + insulin.
[a] $P < 0{\cdot}05$; [b] $P < 0{\cdot}02$; [c] $P < 0{\cdot}001$.

Table 2. Effect of alloxan diabetes and insulin administration on total soluble hexokinase from brain and heart.

	Cerebral hemispheres		Cerebellum		Brain stem		Heart	
Time (days)	D	D + I	D	D + 1 (units/g)	D	D + I	D	D + I
3	$3{\cdot}6^{e} \pm 0{\cdot}3$	3·3 ± 0·8	$3{\cdot}9^{e} \pm 0{\cdot}2$	3·1 ± 0·1	$3{\cdot}3^{e} \pm 0{\cdot}05$	2·8 ± 0·12	$2{\cdot}8^{b} \pm 0{\cdot}1$	3·2 ± 0·2
8	$3{\cdot}6^{a} \pm 0{\cdot}2$	3·2 ± 0·1	$3{\cdot}2^{b} \pm 0{\cdot}1$	2·8 ± 0·1	3·0 ± 0·2	2·8 ± 0·1	$2{\cdot}7^{d} \pm 0{\cdot}1$	3·6 ± 0·2
15	$4{\cdot}1^{e} \pm 0{\cdot}1$	3·6 ± 0·3	$3{\cdot}5^{c} \pm 0{\cdot}2$	$3{\cdot}4^{c} \pm 0{\cdot}3$	$3{\cdot}0^{c} \pm 0{\cdot}1$	$3{\cdot}1^{a} \pm 0{\cdot}2$	3·1 ± 0·2	3·5 ± 0·2
Control	3·1 ± 0·1		2·8 ± 0·1		2·6 ± 0·1		3·6 ± 0·2	

Each value is a mean ± S.E.M. of four or more experiments done in triplicates.
D = Diabetes and D + I = diabetes + insulin.
[a] $P < 0{\cdot}05$; [b] $P < 0{\cdot}02$; [c] $P < 0{\cdot}01$; [d] $P < 0{\cdot}002$; [e] $P < 0{\cdot}001$.

In the cerebellum, total and Type I hexokinase activity from TPF did not change. In the soluble fraction, however, total as well as Type I isoenzyme activity increased significantly after 3, 8 and 15 days (tables 1 and 2). A significant decrease in hexokinase Type II isoenzyme activity was observed at 8 days ($P < 0{\cdot}05$), with a subsequent increase in the soluble form at 8 and 15 days ($P < 0{\cdot}05$ and $P < 0{\cdot}01$, respectively). Results are given in tables 1 and 2 and figure 1B.

The total and Type I hexokinase activity from particulate fraction in the brain stem did not show any change with diabetes, but the enzyme activity in the soluble fraction increased significantly at 3 and 15 days after alloxan treatment. Hexokinase Type II activity in TPF showed a significant decrease at 3 days ($P < 0{\cdot}05$) with no change in the soluble form. The results are presented in tables 1 and 2 and figure 1C.

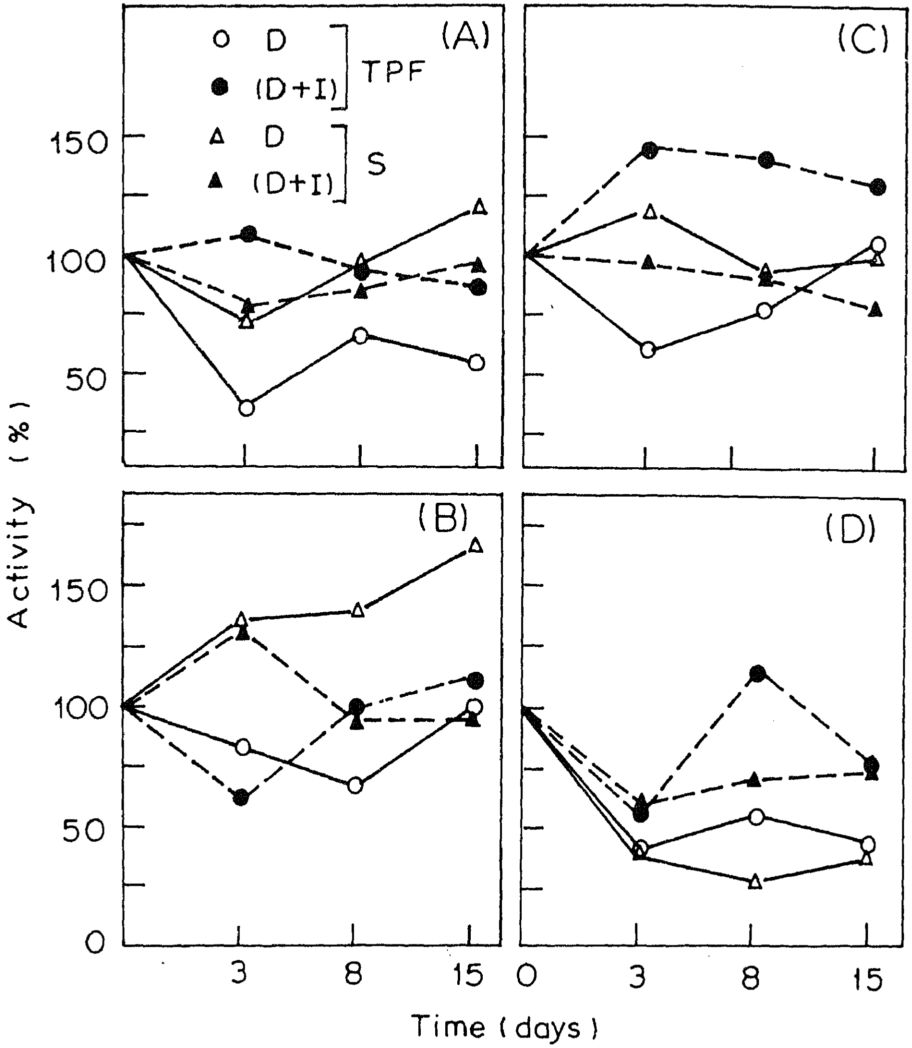

Figure 1. Effect of alloxan diabetes and insulin administration on hexokinase Type II isoenzyme in (A) cerebral hemispheres (CH); (B) cerebellum (CB); (C) brain stem (BS) and (D) heart (H). Absolute activities of hexokinase Type II in control rats from TPF are 1·35 ± 0·08; 0·6 ± 0·06; 0·4 ± 0·03 and 0·41 ± 0·07 in CH, CB, BS and H respectively and from soluble fraction are 0·8 ± 0·04; 0·5 ± 0·06; 0·45 ± 0·05 and 1·3 ± 0·1 in CH, CB, BS and H, respectively. (S, Soluble; D, Diabetes, D + I, diabetes + insulin).

Changes in hexokinase activity in heart tissue

TPF and soluble fraction at all the three time intervals ($P < 0{\cdot}05$ and $P < 0{\cdot}01$, respectively). Administration of insulin to diabetic animals restored the activity of total and Type I hexokinase in heart tissue to near control levels. Results are shown in tables 1 and 2 and figure 1D.

As hexokinase Type I followed the same pattern of changes as total enzyme activity, results of total and Type II isoenzyme are presented in tables and figure, respectively.

Discussion

Significant changes in the intracellular distribution of brain hexokinase with alloxan diabetes were observed during the present investigation. The increase in the activity of the soluble hexokinase found in our experiments may be due to the partial solubilization of the particulate hexokinase, occurring as a consequence of the increased levels of glucose-6-phosphate in the rat brain (Blackshear and Alberti, 1974; Thurston *et al.*, 1975). Wilson (1968) has reported that *in vivo*, the soluble:mitochondrial hexokinase equilibrium is very much sensitive to metabolite control, in particular to glucose-6-phosphate level.

Previous kinetic studies from bovine brain hexokinase have shown that mitochondria-bound hexokinase has a higher K_i for glucose-6-phosphate and a lower K_m for ATP as compared to the soluble form, suggesting that the bound form of the hexokinase in brain is the more active form (Copley and Fromm, 1967). The results obtained in the present experiments, therefore, suggest that the more active form (particulate) of hexokinase in the rat brain, may be converted to a less active (soluble) form under conditions of reduced cerebral metabolism.

The increased levels of free fatty acids in the brain during hypoxia cause solubilization of the brain hexokinase *in vivo* (Domanska-Janik *et al.*, 1978). Diabetes leads to the increased levels of free fatty acids in the blood (Schonfeld and Kipnis, 1968; Hall *et al.*, 1976), which are permeable through the blood brain barrier (Galli *et al.*, 1971). The decreased activity of hexokinase in the particulate fraction of brain with diabetes may partly be a consequence of the solubilizing action of free fatty acids on particulate bound hexokinase with an increase in the soluble enzyme activity.

Katzen (1967) has reported that Type II isoenzyme is the adaptive form of hexokinase and is affected more in diabetes, as compared to Type I, in insulin sensitive tissues such as heart and adipose tissue. During the present experiments, hexokinase Type II, although present in very low amounts (15–20%) in brain, showed more significant changes than Type I and followed similar pattern of changes as that in the heart tissue.

Insulin administration reverses the effect of alloxan diabetes on hexokinase activity. The recovery in the enzyme activity may be due to the decrease in the free fatty acids level after insulin administration (Hall *et al.*, 1976), and correction in most of the metabolite changes seen in the brain of alloxan diabetic rats, especially glucose-6

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Rapid calorie metering in *ad lib* rats

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Abstract. Adult wistar rats of either sex housed in individual cages and fed *ad lib* were used to investigate the effects of calorie content and taste on ingestion. A 15 min single-choice solution (water, quinine, sucrose, saccharin) test as well as 1 h mixed diet (stock diet, sucrose-mixed, saccharin-mixed, quinine-mixed) tests were used. Calorically-rich sucrose either in solution or in mixed-diet was preferentially ingested. Taste *per se* has not influenced calorie intake, as intake on sweet saccharin (3·4 ± 0·4 cal) or on bitter quinine (3·3 ± 0·6 cal) was similar to intake on stock diet (4·8 ± 0·8 cal). Increased 5 min intake on sucrose solution (4·6 ± 0·1 ml) over intake of other test solutions (saccharin 3·5 ± 0·2 ml, quinine, 1·2 ± 0·3 ml, water 2·1 ± 0·1 ml) and calorie intake suppression on 1 h stock diet immediately following sucrose solution intake, indicate rapid calorie metering, probably based on fast-acting specific gustatory signals.

Keywords. Taste; calorie-intake.

Introduction

Recent review of food intake mechanisms (Le Magnen, 1983) has shown that 24 h calorie intake of the free feeding animal is maintained constant (Le Magnen and Devos, 1980) and that any deviation from this constancy is adjusted in 6 h period (Booth, 1972). The post-absorptive metabolic factors such as glucose (Mayer, 1953) and free fatty acids (Oomura, 1976) are known to act on central chemoreceptive mechanisms (Anand, 1967) and thus help in regulation of 24 h calorie intake. However daily calorie requirement is obtained *via* intermittent and discreet meals though rats being nibblers have high frequency of meals per day (18–26) with meal duration (4·3–4·9 min) and meal size per rat (1·6–1·7 g) being small (Strubbe *et al.*, 1977). As meal intake time is short, termination of eating at the end of each meal cannot be due to post-absorptive metabolic effects, but may be resultant of distension of stomach (Sharma *et al.*, 1961), effects of taste (Jacobs and Sharma, 1969) or olfaction (Le Magnen, 1969). Whether these intra meal intake regulators also regulate calories is a mute point. Among those intake regulators taste is known to be correlated with metabolic cues (Le Magnen, 1967) and hence its effect as well as the effects of calorie content in food on intake of free feeding rats in very short periods are investigated.

Materials and methods

Adult wistar rats (n = 27; male 20; female 7) aged 4–5 months, housed in individual cages were used. The body wt of male rats (259·6 ± 30·6 g) was higher than body wt of female rats (200·9 ± 10·3 g). Stock diet prepared as per Rao and Sharma (1980) and tap water were provided *ad libitum.* After 10 days of adaptation to feeding and drinking in their home cages the food intake was measured at regular intervals of 2 h for 3 day period. Then the following tests were conducted on all animals. On any day solution test was followed by either stock diet or mixed diet test for 1 h. For the rest of 23 h, stock diet was given.

Solution tests

Tap water and solutions of sucrose (9 %); saccharin (0·2 %) and quinine (0·002 %) made in tap water were used. The cage was cleared of food and water bottles during solution test. Only one test solution was given on any one day at 1730 h (5:30 p.m.) and its intake in initial 5 min and at the end of 15 min period were noted. Water intake served as control.

Pre-meal taste effects

The effects of taste and ingestion of test solutions on calorie intake in 1 h (1800–1900 h) and 24 h stock diet was measured. Water was available during the stock diet intake.

Mixed diet test

Sucrose-mixed (10 %); saccharin-mixed (0·1 %) and quinine-mixed (0·5 %) diets were prepared as described in Rao and Sharma (1980). Taste substance was dissolved in water before its addition to powder diet. Stock diet intake in 1 h served as control.

After the tests, *ad libitum* stock diet and water were given for the day. The intake of test solutions (ml) and calorie intake on stock and mixed diets are expressed per 100 g body wt. Student '*t*' test is used for statistical analysis and $P < 0·05$ is taken as significant difference.

Results

Diurnal eating rhythms of the rats observed at 2 h period intervals showed that intake was maximal (4·8 ± 0·6 cal) during 1800–2000 h period, whereas in other periods of observation the intake ranged from 0·0–2·9 cal. One-hour mixed diet tests indicated (figure 1) that intake on saccharin diet (3·4 ± 0·4 cal) and bitter quinine diet (3·3 ± 0·6 cal) is similar to calorie intake on stock diet (4·8 ± 0·8 cal) whereas intake on sucrose diet (10·8 ± 1·2 cal) increases significantly. Preferential intake of calorically rich sucrose over sweet but calorically inert saccharin was substantiated by solution tests (table 1). Though 15 min intake of sucrose solution (5·8 ± 0·5 ml) and saccharin (4·2 ± 0·6 ml) were significantly increased over water intake (2·3 ± 0·1 ml) the increase was more on sucrose than on saccharin. It may be noted that in initial 5 min period, 80 % or more of total 15 min intake is already ingested. The intake in rest of 10 min period is more on sucrose as compared to intake on other test solutions. Further, after sucrose solution intake and not after intake of other test solutions a decrease in calorie intake

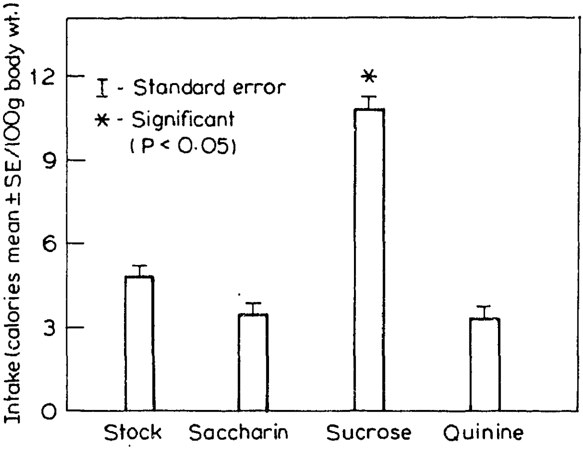

Figure 1. Test-diet intake (begin 1 h) of *ad lib* rats.

Table 1. Solution intake in rats fed *ad lib.*

Test solution	Intake in initial 5 min	Intake in terminal 10 min	%5 min intake over 15 min intake
Water	2·1 ± 0·1	0·2 ± 0·1	91·2
Quinine	1·2 ± 0·3	0·1 ± 0·05	92·2
Saccharin	3·5 ± 0·2	0·7 ± 0·3	83·1
Sucrose	4·6 ± 0·1	1·2 ± 0·3	79·3

The values are expressed as ml/100 g body wt and represent the mean ± S.E. from 27 rats.

Table 2. Calorie intake of rats fed *ad lib.*

Pre-meal taste solution	Calories on solution	Calories on stock diet in 1 h	Calories on stock diet in 24 h
Water	0·0	4·8 ± 0·8	19·7 ± 1·9
Quinine	0·0	6·2 ± 0·1	20·1 ± 1·0
Saccharin	0·0	6·3 ± 0·4	19·8 ± 1·1
Sucrose	2·2	2·9 ± 0·1	21·3 ± 1·2

The effects of taste and calorie content of food on short-term calorie intake regulation of *ad libitum* rats are investigated. The intake investigations were confined to 1800–2000 h period as diurnal intake of the rats used here showed maximal food intake at that period of the day. Brief solution tests were used earlier (Cagan and Maller, 1974) and are known to indicate taste-based preference (Booth and Brookover, 1968) and hence used here. One-hour mixed diet test in addition to 15 min solution tests were intended to indicate pre-absorptive and possibly taste-related calorie detection and calorie compensations.

The evidence from solution tests of the present investigation that *ad lib* rats increase their intake on calorically-fortified sweet sucrose rather than on sweet but calorically-inert saccharin in a brief 5 min period indicated the existence of pre-absorptively effective rapid calorie detection system. Further, the appropriate reduction in 1 h stock diet intake following the intake of sucrose solution but not after the intake of calorically-inert solutions, including sweet saccharin, reinforced the idea of rapid calorie detection. As the intake of sucrose solution is more than the intake of other test solutions, stomach distension-induced satiety (Sharma *et al.*, 1961) may also be thought of as a causative factor for reduction in 1 h intake but it is not tenable because gastric emptying time of sucrose is known to be approximately 20 min (Elias *et al.*, 1968). Yet another finding that 1 h calorie intake on calorically-identical mixed diets (quinine-mixed and saccharin-mixed) is similar to the intake on stock diet indicated that calories are somehow identified rapidly. The notable exception to constancy of 1 h calorie intake is the intake on sucrose-mixed diet which is increased as compared to intake on other test diets. This probably is the result of *ad lib* rats biasing system for calories (Jacobs and Sharma, 1969) being influenced by rapidly relayed messages of calorie content. Obviously the facilitation of eating for calories is effective for short pre-absorptive period of approximately 1 h, as 24 h calorie intake after sucrose diet also is similar to the intake on other test diets and stock diet.

The mechanisms for rapid calorie detection are not investigated here. However from the data of other reports two possible mechanisms are suggested. One is the pre-absorptive anticipatory humoral signals such as increased blood glucose and insulin secretion on food intake. But as time-course of increase in blood glucose levels (Nicolaidis, 1969) and insulin secretion (Strubbe *et al.*, 1977) show a latency of more than 5 min period, the humoral signals may not be useful for rapid (within 5 min) detection of calories. These signals as well as gastric chemoreceptor activity (Deutsch and Wang, 1977) probably help as indicators of calories during 1 h food intake. Alternatively as gustation is faster acting signalisation of food than the humoral signals the gustation is suspected to be the basis for rapid detection of calories. Further, the taste signals of sucrose and saccharin are known to be distinctly different (Gordon *et al.*, 1956) and possibly help in fast discrimination between the two substances. Either by long association of taste with post-absorptive metabolic consequences (Le Magnen, 1967) or due to innate taste hedonics (Pfaffman, 1964) the animals rapidly recognise the calorie content of ingesta.

Acknowledgement

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Evaluation of mutagenicity of the antitubercular naphthylglycine hydrazides in *Salmonella typhimurium*

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Abstract. N-(2-naphthyl)glycine hydrazide and N-methyl-N-(2-naphthyl) glycine hydrazide, which inhibit *Mycobacterium tuberculosis* $H_{37}R_v$ and show activity against experimental tuberculosis, were evaluated for their mutagenic potential in *Salmonella typhimurium*. Both the compounds at concentration ranges from 0·1 μg/plate to 1000 μg/plate failed to induce mutations at the histidine locus either directly or after treatment with rat liver homogenate fraction–"S-9". N-(2-naphthyl)glycine hydrazide and its N-methyl derivative elicited toxicity at concentrations of 500 μg/plate and 1000 μg/plate. However, in the presence of the liver homogenate system, reduction in toxicity was noticed probably due to detoxification and/or conjugation of the compounds. Under the assay conditions employed, standard mutagens like 4-nitroquinoline-N-oxide, 9-aminoacridine and benzo(*a*)pyrene were positive. The non-mutagenic nature of N-(2-naphthyl)glycine hydrazide and N-methyl-N-(2-naphthyl)glycine hydrazide should enhance their potential for inclusion in treatment protocols for management of tuberculosis.

Keywords. N-(2-naphthyl)glycine hydrazide; N-methyl-N-(2-naphthyl) glycine hydrazide; *Salmonella typhimurium* mutagenicity; antitubercular hydrazides.

Introduction

Tuberculosis continues to be one of the major health problems in India. The main reason for this has been the emerging resistance of *Mycobacterium tuberculosis*, the causative organism of this communicable disease to currently available antitubercular drugs. In an attempt to screen newer drugs active against such organisms we have synthesized two promising compounds, N-(2-naphthyl)glycine hydrazide and N-methyl-N-(2-naphthyl)glycine hydrazide (Ramamurthy, 1983). Earlier studies with N-(2-naphthyl)glycine hydrazide indicated lesser toxicity of this compound in mice compared to the widely used drug, isoniazid (Ramamurthy *et al.*, 1978). N-(2-naphthyl)glycine hydrazide and its N-methyl derivative inhibited *M. tuberculosis* $H_{37}R_v$ *in vitro* and were active against experimental tuberculosis in guinea pigs (Ramamurthy, 1983). However, in view of the structural similarity of these compounds to naphthylamine, a well-known carcinogen, the present studies were undertaken to glycine hydrazide and N-methyl-N-(2-naphthyl)glycine hydrazide do not have a carcinogenic potential.

Materials and methods

Materials

Benzo(*a*)pyrene was obtained from Aldrich and 4-nitroquinoline-1-oxide from Schuchardt (Munich). NADP, 9-Aminoacridine, glucose-6-phosphate were purchased from Sigma Chemical Co., St. Louis, Missouri, USA. Aroclor 1254 was supplied by Analabs, Inc., North Haven, Connecticut, USA. All other chemicals were of analytical grade available commercially.

N-(2-naphthyl)glycine hydrazide was prepared according to the procedure of Tien *et al.* (1958). The N-methyl derivative of the above compound was also synthesized (Ramamurthy, 1983). Both the compounds were crystallised to obtain chromatographically pure compounds.

Organisms, media and culture conditions

Salmonella typhimurium strains TA 1537 (histidine mutation *his C3076*), TA 98 (histidine mutation *his D3052* and R factor plasmid *pKM 101*) and TA 100 (histidine mutation *his C46* and R factor plasmid *pKM 101*) were used in the present studies. Each tester strain also carries two additional mutations, one which causes loss of the excision repair system (uvrB) and the other loss of the lipopolysaccharide barrier (rfa) that coats the surface of the bacteria (Ames *et al.*, 1973). The deleted genes due to uvrB are similar to those reported earlier (Ames *et al.*, 1975).

Manipulations of *S. typhimurium* strains were carried out essentially as recommended by Ames *et al.* (1973). Frozen stocks were stored at −80°C and the working copy from which subcultures were obtained was kept at −20°C. To 5 ml of nutrient broth, a small sample of bacteria from the working copy was inoculated and incubated for 12–16 h at 37°C, yielding 10^9 cells/ml. Each subculture was tested for histidine auxotrophy by streaking on minimal nutrient plates with and without histidine. They were also tested for crystal violet inhibitory action to indicate the presence of rfa mutation. The presence of R factor in strains TA 98 and TA 100 was monitored by their resistance to ampicillin.

Preparation of liver homogenate fraction ("*S-9*")

For checking the metabolic activation of the test compounds, incubation with "S-9" fractions were carried out. "S-9" fractions from Aroclor 1254 induced rats were prepared (Ames *et al.*, 1973) and the "S-9 mix" preparations made for metabolic activation of the compounds.

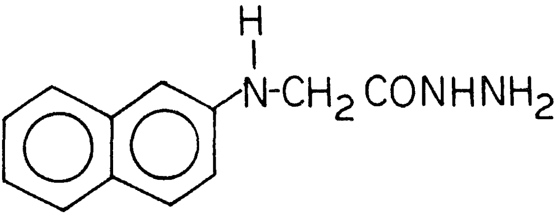
pounds, except 9-aminoacridine were dissolved and serially diluted in dimethylsulphoxide to obtain the appropriate test concentration. The direct mutagen, 9-aminoacridine was dissolved and serially diluted in ethanol.

Mutagenicity assay

Mutagenicity testing was done by scoring for histidine prototrophs as described by Ames *et al.* (1975). Appropriate positive controls namely 4-nitroquinoline-N-oxide and 9-aminoacridine (direct mutagens) and benzo(*a*)pyrene (procarcinogen) were used. Spontaneous reversion frequency was monitored by carrying out the assay in the absence of the compounds.

Results and discussion

It is essential to evaluate the biohazard nature of chemicals having potential as chemotherapeutic agents in order to work out the risk-benefit situation for the use of these compounds. N-(2-naphthyl)glycine hydrazide and N-methyl-N-(2-naphthyl)glycine hydrazide (figure 1) which have inhibitory activity against *M.*



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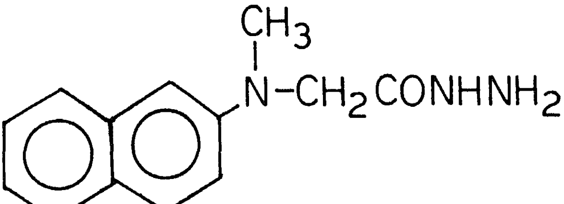

tuberculosis $H_{37}R_v$ and even some of the drug-resistant clinical isolates (Ramamurthy, 1983) are likely candidates for inclusion in the treatment protocols for management of tuberculosis. The added advantage for employing these hydrazides is the reduced toxicity of these compounds as compared to isoniazid (Ramamurthy *et al.*, 1978). The *S. typhimurium* mutagenicity system provides an efficient pre-screen to detect potentially hazardous chemicals (McCann *et al.*, 1975). N-(2-naphthyl)glycine hydrazide in a concentration range of 0·1 μg/plate to 1000 μg/plate failed to elicit mutagenicity in TA 1537, TA 98 and TA 100 strains. At 500 μg/plate and 1000 μg/plate, toxicity (not mutagenicity) was apparent. The positive controls 4-nitroquinoline-N-oxide and 9-aminoacridine at concentrations of 5 μg/plate and 10 μg/plate respectively induced mutagenesis in the *S. typhimurium* strains (table 1). With the addition of "S-9 mix", no metabolic activation with subsequent mutagenicity was apparent in the concentration range of 0·1 μg/plate to 1000 μg/plate for the test compound (table 2). However, with the activation system, toxicity to the bacterial strains was evident only at 1000 μg/plate. The lack of toxicity with "S-9" at 500 μg/plate and reduced toxicity at 1000 μg/plate (as compared to the assay without the activation system) indicates probable detoxification of the compound to less toxic metabolites. N-methyl-N-(2-naphthyl)glycine hydrazide showed no mutagenic activity at the same concentration as that of N-(2-naphthyl)glycine hydrazide (table 3). The pattern of toxicity of this compound at 500 μg/plate and 1000 μg/plate was similar to that observed in the case of N-(2-naphthyl)glycine hydrazide. Even with the "S-9" activation system, the methyl derivative failed to indice mutations in TA 1537, TA 98 and TA 100 strains (table 4).

The present studies point to the conclusion that the naphthyl glycine hydrazides exhibiting anti-tubercular properties are non-mutagenic. The negative results using the

Table 1. Bacterial mutagenicity of N-(2-naphythyl)glycine hydrazide in *S. typhimurium* strains.

Compounds	Concentration (μg/plate)	Histidine revertants per plate		
		TA 1537	TA 98	TA 100
N-(2-naphthyl)glycine hydrazide	0·1	12	47	165
	1·0	10	42	172
	10·0	13	47	170
	100·0	10	45	161
	500·0	4	21	78
	1000·0	0	5	21
Control (no compounds)	—	8	42	162
4-Nitroquinoline-N-oxide (positive control)	5·0	15	1236	3862

Table 2. Metabolic activation of N-(2-naphthyl)glycine hydrazide and mutagenesis in *S. typhimurium* strains.

Compounds treated with "S-9 mix"	Concentration (μg/plate)	Histidine revertants per plate		
		TA 1537	TA 98	TA 100
N-(2-naphthyl)glycine hydrazide	0·1	18	67	192
	1·0	17	70	197
	10·0	14	73	184
	100·0	14	70	191
	500·0	15	64	181
	1000·0	5	23	96
Control (no compounds)	—	12	54	176
Benzo(*a*)pyrene (positive control)	5·0	827	2187	2248

Table 3. Evaluation of mutagenicity of N-methyl-N-(2-naphthyl) glycine hydrazide using *S. typhimurium* strains.

Test compound	Concentration (μg/plate)	Histidine revertants per plate		
		TA 1537	TA 98	TA 100
N-Methyl-N-(2-naphthyl) glycine hydrazide	0·1	11	53	177
	1·0	14	48	174
	10·0	10	56	178
	100·0	9	41	162
	500·0	2	28	96
	1000·0	0	12	46
Control (no compounds)	—	9	45	167
4-Nitroquinoline-N-oxide (positive control)	5·0	16	1247	3852
9-Aminoacridine (positive control)	10·0	805	83	226

liver homogenate indicate that the probability of these compounds culminating as mutagens, in a manner similar to the procarcinogen – benzo(*a*)pyrene (Ames *et al.*, 1975; McCann *et al.*, 1975), is very less. Our earlier study with certain compounds which are direct mutagens has shown that drug-metabolizing enzymes can detoxify and convert these chemicals to non-mutagens (Pai, 1983). Aslo detoxification of compounds by liver can generate metabolites that are not genotoxic (Pai *et al.*, 1983).

Table 4. Mutagenesis of N-methyl-N-(2-naphthyl)glycine hydrazide in *S. typhimurium* subsequent to metabolic activation.

Test compounds treated with "S-9 mix"	Concentration (μg/plate)	Histidine revertants per plate		
		TA 1537	TA 98	TA 100
	0·1	12	63	193
	1·0	18	71	197
N-Methyl-N-(2-naphthyl) glycine hydrazide	10·0	16	68	188
	100·0	16	77	196
	500·0	11	78	201
	1000·0	4	33	111
Control (no compounds)	—	11	52	172
Benzo(*a*)pyrene (positive control)	10·0	809	2106	2231

search for better drugs is to eliminate the mutagenic and probable carcinogenic nature of the compounds while retaining the desirable biological property (Venger *et al.*, 1979; Harfenist, 1980). The non-mutagenic nature of the naphthylglycine hydrazides used in the present study coupled to reduced toxicity in experimental animals should enhance their potential for use as effective antitubercular drugs.

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alloxan diabetic rats

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Abstract. Diabetes intensifies the development of atherosclerosis. Treatment with antihypertensive drug, serpasil, arrested the progression of atherosclerosis in alloxan diabetic rats by significantly decreasing the concentration of cholesterol, phospholipids and triglycerides of serum, liver, kidney and aorta. Serpasil also decreased fasting blood sugar and urine sugar levels in these rats. Serpasil administration remarkably altered the deranged lipid metabolism in alloxan diabetic rats by nearly restoring the lipolytic and lipogenic enzyme activity to that of the normal.

Keywords. Diabetes; hypotensive agent; serpasil; lipoprotein lipase; lipogenic enzymes.

Introduction

It is now well accepted that the risk of vascular disease is increased due to diabetes. Serpasil (reserpine) a hypotensive agent, used both for normal and diabetic hypertension is reported to reduce the incidence of aortic rupture in turkeys and in human beings (Palmor *et al.*, 1965). Carrier *et al.* (1968) reported that serpasil inhibited cholesterol induced vascular lesions in experimental animals.

The hypotensive activity of serpasil is well established and it is known to lessen the severity of atheromatous lesions and prevents high blood levels of cholesterol in animals fed on a diet containing a high quantity of cholesterol. However, its effect on the lipid metabolism of diabetic rat, to the best of our knowledge, has not been studied. Therefore, the effects of serpasil on lipid metabolism in alloxan diabetic rats have been studied.

Materials and methods

Male albino rats (Sprague Dawley Strain 100–150 g) were made diabetic by the injection of alloxan (Prahl *et al.*, 1965). The diabetic animals having blood sugar levels between 200–250 mg/dl were selected for the study. They were divided into two groups of 6 each, one for diabetic control and the other for serpasil treatment. Two groups of normal rats with same average weight were selected, one maintained as normal and the other treated with serpasil. Urine was collected from all the groups and its sugar content estimated (Benedict, 1911). Before the drug treatment, an initial fasting blood sugar level was determined in all the animals. Serpasil (CIBA-GEIGY, Bombay) was given

Table 2. Concentration of lipids in serum and tissue of normal and diabetic rats treated with serpasil.

Group	Total cholesterol	Phospholipids	Triglycerides glycerol	Total cholesterol	Phospholipids	Triglyceride glycerol
		Serum			Liver	
1	68·04 ± 1·34	126·4 ± 3·45	7·40 ± 0·14	342·6 ± 5·2	2546 ± 44·2	484·4 ± 10·5
1a	71·23 ± 1·45	120·8 ± 3·62	7·12 ± 0·19	321·8^{c} ± 4·75	2590 ± 48·8	443^{c} ± 14·1
2	184·34^{a} ± 3·90	238·0^{a} ± 4·20	23·4^{a} ± 1·4	430·3^{a} ± 6·2	3538^{b} ± 50·3	682·0^{a} ± 12·2
3	75·00^{a} ± 1·52	143·0^{a} ± 3·70	9·63^{a} ± 0·16	360·5^{a} ± 4·5	2386^{a} ± 31·3	523^{b} ± 10·6
		Aorta			Kidney	
1	167 ± 3·3	943·2 ± 25·8	1103 ± 22·1	287 ± 10.6	2162 ± 41·8	98·5 ± 1·8
1a	161·2 ± 3·71	864·7 ± 28·1	1058 ± 20·1	263·1 ± 12·1	2018^{c} ± 38·2	94·6 ± 1·79
2	685^{a} ± 8·2	1886·4^{b} ± 63·2	2368^{b} ± 98·3	364^{b} ± 8·3	3692^{a} ± 53·16	166^{a} ± 5·8
3	172^{a} ± 3·2	956·4^{b} ± 26·2	1112^{b} ± 22·4	292^{b} ± 9·4	2371^{a} ± 32·67	103^{a} ± 2·1

Average of the values from 6 rats in each group ± S.E., expressed as mg/dl serum and mg/100 g wet tissue
Group 1. normal, Group 1a serpasil treated Normal, 2. diabetic, 3. serpasil treated diabetic
1a is compared with 1.
2 is compared with 1 and 3.
[a] $P < 0{\cdot}001$.
[b] $P < 0{\cdot}01$.
[c] $P < 0{\cdot}05$

Table 3. Lipoprotein lipase activity in serpasil treated rats.

Group	Aorta	Liver	Heart	Adipose
1	221·3 ±6·5	24·3 ±0·95	39·5 ±0·89	185·3 ±6·0
2	185·7^{b} ±3·3	20·4^{b} ±0·72	44·6^{a} ±0·91	260·4^{a} ±7·2
3	223·1^{a} ±4·7	23·2^{b} ±0·86	38·2^{a} ±0·85	182·4^{a} ±5·3

Activity expressed in μ mol glycerol liberated/h/g protein are mean ± S.E. from 6 rats. Foot notes same as in table 2.

body weight was not observed in serpasil administered diabetic animals during the month. In contrast, the body weight of the serpasil treated animals was significa higher than the diabetic control rats. The fasting blood sugar and urine sugar l significantly decreased in the serpasil treated group as compared with the signifi increase in the diabetic control group. No significant difference was observed in no rats treated with serpasil.

The results presented in table 2 indicate that the values of total cholest phospholipids and triglycerides in serum, liver, aorta and kidney of diabetic ani were significantly elevated than the controls. Treatment with serpasil remark altered these parameters. The values significantly decreased were cholesterol in se liver and aorta, phospholipids in serum, liver and kidney, triglycerides in serum, a and kidney. Serum and tissue lipid levels were not significantly altered in normal treated with serpasil.

The results presented in table 3 indicate that the lipoprotein lipase activity incre in heart and adipose tissue, and decreased in aorta and liver of diabetic animals. S no protamine has been used in these studies, the activity measured includes in add to lipoprotein lipase, some triglyceride lipase also. Serpasil administration alt these conditions by restoring lipolytic activity to that of the normal control. activities of lipogenic enzymes in liver and adipose tissue are presented in table 4. T was significant decrease in the activity of all lipogenic enzymes in liver and adi tissue of diabetic rats. Administration of serpasil ameliorated the diabetic cond and hence significantly increased these levels.

Although considerable damage to the pancreas occurred in alloxan diabetic ani serpasil has been found to decrease the fasting blood sugar and urine sugar in these and it may be due to its hypoglycemic effect. Decreased lipoprotein lipase activ associated with increased accumulation of lipids (Seethanathan *et al.*, 1970). increased accumulation of lipids, especially triglycerides, in tissues such as liver aorta in alloxan diabetic rats is in agreement with this proposition. It is observed serpasil treatment increases the decreased lipolytic activity in these tissues. depressed lipogenic enzyme activity in alloxan diabetic rats is brought back near normal by the administration of serpasil. Increased levels of cholesterol in serum tissues of alloxan diabetic rats are significantly decreased by the intake of serpasi

Table 4 Activity of lipogenic enzymes of liver and adipose tissue in serpasil treated rats

	Liver			Adipose		
Enzyme	Group 1	Group 2	Group 3	Group 1	Group2	Group 3
Glucose-6-phosphate dehydrogenase units/g protein	107·3 ± 2·9	79·5^{a} ± 3·1	102·4^{a} ± 2·3	29·3 ± 1·4	12·2^{a} ± 0·42	29·1^{a} ± 1·2
6-Phosphogluconate dehydrogenase units/g protein	125 ± 5·6	102·5^{c} ± 3·8	116·4 ± 5·2	31·6 ± 1·5	13·8^{a} ± 0·5	31·2^{a} ± 1·3
Isocitric dehydrogenase units/mg protein	1·564 ± 0·04	0·974^{a} ± 0·06	1·365^{a} ± 0·05	105·7 ± 4·1	33·7^{a} ± 1·6	115·3^{a} ± 4·2
Malic enzyme units/mg protein	1·52 ± 0·05	1·12^{a} ± 0·02	1·46^{a} ± 0·04	49·9 ± 1·9	17·9^{a} ± 0·6	51·2^{a} ± 2·1
Malic dehydrogenase units/mg protein	8·52 ± 0·24	5·92^{a} ± 0·12	7·83^{a} ± 0·22	46·5 ± 1·65	16·5^{a} ± 0·6	45·3^{a} ± 1.4

Values are mean ± S.E. from 6 rats.
Foot note same as in table 2.

pholipids decreased in serum and tissues of serpasil treated rats, the maximum being in serum, liver and kidney. Since this drug deranges or corrects the abnormalities observed in the lipid metabolism of alloxan diabetic animals, extended investigations are warranted to establish its mechanism of action.

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Uridine 5′-diphosphate glucose 4-epimerase from Ehrlich ascites carcinoma cells

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Abstract. Uridine 5′-diphosphate glucose 4-epimerase (EC 5.1.3.2) from Ehrlich ascites carcinoma cells was purified to apparent homogeneity using conventional procedures and NAD-hexane-agarose affinity chromatography. The protein had a molecular weight of 96,000. The ascites enzyme had an absolute requirement for exogenously added NAD (10 μM) for stability. This appears to be a unique feature of ascites epimerase since epimerase from other mammalian sources did not exhibit such a dependence. Exogenously added NAD was also needed for catalysis with an apparent K_m value of 2·5 μM. NADH was a very potent competitive inhibitor ($K_i = 0{\cdot}11$ μM with respect to NAD) of the enzyme activity at pH values close to intracellular pH. The dependence of the enzyme on NAD for stability and its inhibition by NADH may have some potential significance in tumor metabolism.

Keywords. Galactose metabolism; UDP glucose 4-epimerase; tumor.

Introduction

Galactose is an essential component of mammalian cell-surface glycoconjugates. Faithful expression and biosynthesis of some of these membrane glycoconjugates have often been implicated in the dramatic changes that are brought about at the cell surface during neoplastic transformations and in the subsequent metastatic processes (Rapin and Burger, 1974; Nicolson, 1982). Galactose being an integral component of such glycoconjugates, its synthesis in the activated nucleotide-sugar form and its subsequent transfer to the acceptor molecules may, therefore, have significant bearing on the development of malignancy or its invasiveness.

Work on galactose metabolism and related enzymes of this pathway, both in clinical and with experimental carcinoma, have indicated that significant alterations in the enzyme pattern take place on neoplastic transformation. Level of serum galactosyl transferase have been tentatively used as a diagnostic tool in ovarian carcinoma, though the origin of the transferases still remains uncertain (Chatterjee *et al.*, 1979, 1980). Sera of patients with other types of cancer have also been shown to have elevated levels of this enzyme (Weiser *et al.*, 1976), though contradictory reports have also appeared in

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Abbreviations used: UDP, Uridine 5′-diphosphate; buffer A, sodium phosphate buffer, pH 7·4, 20 mM, containing 2-mercaptoethanol 1 mM, EDTA 1 mM, NAD 10 μM; DEAE, diethylamino ethyl; EDTA, ethylene diamine tetra acetic acid (disodium salt).

the literature (Ip and Dao, 1978). Eagle *et al.* (1958) had originally observed that the growth of some tumor cells in culture media was severely affected when galactose replaced glucose as the sole carbon source. Radioisotopic studies on the flux of galactose through the Leloir pathway by Kalckar's group had shown uridine 5′-diphosphate (UDP) glucose 4-epimerase to be the rate limiting enzyme in cultured tumor cells and studies on the crude enzyme from HeLa cells indicated some regulatory potential for this enzyme (Robinson *et al.*, 1966). Subsequently Mitchell *et al.* (1975) claimed that phytohemagglutinin-stimulated lymphoblast cultures derived from leukocytes of individuals lacking in epimerase showed significant levels of renewed epimerase activity inspite of the original deficiency. Recently, working with a number of solid Morris hepatomas Bauer *et al.* (1980) have shown that the activity of the epimerase is elevated several fold in rapidly growing and poorly differentiated cells. In view of the above reports and since the epimerase is known to be critically involved in maintaining the UDP galactose pool either with glucose or with galactose as the starting substrate (Kalckar, 1965), we decided to characterize the enzyme extensively from a suitable tumor source.

UDP glucose 4-epimerase (EC 5.1.3.2) has been purified and characterized in detail from several mammalian sources including calf liver (Geren and Ebner, 1977; Geren *et al.*, 1977; Langer and Glaser, 1974) and lactating bovine mammary gland (Geren *et al.*, 1977). Extensive mechanistic studies on this enzyme has been carried out with homogeneous enzymes obtained from *Escherichia coli* or from *Saccharomyces fragilis* (Gabriel *et al.*, 1975). No report has, however, been published regarding the purification and characterization of the enzyme from any tumor source. In this work, we report the purification to apparent homogeneity and partial characterization of this important enzyme of galactose metabolism from ascites tumor cells.

Materials and methods

All the biochemicals including the enzymes, unless otherwise stated, were purchased from Sigma Chemical Company, St. Louis, Missouri, USA. NAD-hexane agarose (Type 1, No. 5485) was a product of P. L. Biochemicals. Calcium phosphate gel was prepared according to the method of Keilin and Hartree (1938).

UDP glucose 4-epimerase was routinely assayed with UDP-galactose as the substrate in a coupled assay system that allowed continuous monitoring of the formation of NADH in the presence of UDP glucose dehydrogenase and added NAD (Ray and Bhaduri, 1975). A parallel two step assay was also performed for the determination of epimerase activity whenever it was necessary to assess the role of exogenous NAD on the activity of the enzyme or whenever UDP glucose was used as the starting substrate. This assay has been described previously (Ray and Bhaduri, 1975).

Protein was estimated according to the method of Lowry *et al.* (1951) or by measuring absorbancy at 280 nm, taking in both cases albumin as the standard protein. In some cases where a high concentration of NAD was present in the enzyme fraction, it

After 10–12 days, ascites fluid was collected from mice by syringe. The ascites fluid ontaining the ascites cells was immediately diluted with 5 volumes of cold normal aline to avoid coagulation. Erythrocytes which were found to be occasionally ontaminated with ascites cells were removed by repeated washings with hypotonic aline (0·2 %). The ascites cells at this stage were homogenized for purification of UDP lucose 4-epimerase.

Results

Purification of UDP glucose-4-epimerase from Ehrlich ascites cells

Unless otherwise stated, all operations during purification were carried out at 4°C. As he enzyme was unstable in the absence of added NAD, a solution containing 20 mM odium phosphate buffer, pH 7·4, 1 mM 2-mercaptoethanol, 1 mM EDTA and 10 μM NAD was routinely used during various purification steps. This solution was designated as buffer A.

Sixty gm (wet wt) packed ascites cells were intermittently homogenized in a mortar-pestle with acid-washed sea sand for 10–15 min and extracted with 180 ml of buffer A. The homogenate was centrifuged at 15,000 *g* for 25 min. The fatty layer was removed rom the top and the supernatant was used for further purification. This was termed as rude homogenate.

First ammonium sulphate fraction

olid ammonium sulphate (262 g/L) was slowly added with constant stirring to the bove crude homogenate and after standing for 10 min, it was centrifuged at 15,000 *g* or 15 min. The supernatant was again treated with solid ammonium sulphate (150 g/L). The resulting precipitate was dissolved in 20 ml of buffer A and dialyzed for –7 h against the same buffer (3 L) with two changes. The volume of the dialyzed nzyme became 25 ml.

Purification by calcium phosphate gel

Calcium phosphate gel (22 ml, dry wt 11 mg/ml) was centrifuged at 1000 *g* for 1 min and the supernatant was rejected. To the pellet, 25 ml dialyzed enzyme (36 mg protein/ml) was added with stirring and centrifuged at 4000 *g* for 3 min. To the upernatant (25 mg protein/ml) 11·5 ml of calcium phosphate gel treated as above was added, mixed and centrifuged at 4000 *g* for 3 min. The supernatant was retained. Following a similar treatment of the supernatant (17 mg/ml) with 7·5 ml of calcium phosphate gel for the third time, the supernatant after centrifugation, was retained for urther purification.

Second ammonium sulphate fraction: To 25 ml of the above enzyme fraction solid mmonium sulphate (260 g/L) was slowly added with constant stirring. After standing for 10 min, it was centrifuged at 15000 *g* for 15 min and the precipitate was discar The supernatant was treated in the same manner with solid ammonium sulp (154 g/L) and centrifuged as above. After rejecting the supernatant, the pellet dissolved in 2 ml of 'buffer A' and dialyzed for 6 h against the same buffer.

DEAE-cellulose column

The dialyzed enzyme fraction (2·5 ml) was loaded on a DEAE cellulose column × 30 cm) previously equilibriated with 'buffer A'. The column was then successi washed with 20 mM, 50 mM, 100 mM and 200 mM sodium phosphate buffer, pH each containing 1 mM 2-mercaptoethanol, 1 mM EDTA and 0·01 mM NAD. enzyme was eluted with 200 mM buffer. Flow rate was maintained at 1 ml/4 min. tubes containing high specific activities were pooled and concentrated with s ammonium sulphate at 80 % saturation. After centrifugation at 15000 *g* for 30 mir precipitate was dissolved in 0·5 ml of 'buffer A' and dialyzed for 3 h against 20 sodium phosphate buffer containing 1 mM 2-mercaptoethanol, 1 mM EDTA 0·5 μM NAD. This enzyme fraction was further purified on NAD-hexane aga affinity column on the same day.

Purification on NAD-agarose affinity column

The above fraction was passed through an NAD-hexane agarose column (0·5 × 4 that was equilibriated with 20 mM sodium phosphate buffer containing 1 mM mercaptoethanol, 1 mM EDTA and 0·5 μM NAD. The rate of flow was 1 ml/20 fractions (0·5 ml) were collected. Unadsorbed proteins were washed out of the col with the same buffer, which was at least 5 times the volume of the column. Two diffe concentrations of NAD, 1 mM and 10 mM along with the buffer were used fo elution of the adsorbed proteins. Bulk of the bound protein was eluted with 1 NAD but the epimerase which was apparently more tightly bound could be eluted with the buffer containing 10 mM NAD. The yield was approximately 30 % o amount applied to the column. The final yield over the crude fraction was gene between 8 and 10 % and the enzyme was purified about 11,000 fold over the c fraction. The yield and fold of purification during the various stages of purificatio presented in table 1.

Homogeneity and molecular weight

The highly purified enzyme obtained from the affinity column was concentr approximately to one fifth of the original volume against solid sucrose. Polyacryla gel electrophoresis with 7·5 % and 10 % cross-linking and at pH 7·4 and at pH revealed in each case, the presence of one clear single band. To confirm the presen epimerase activity in this band, a thin gel slice (the buffer for electrophoresis conta in addition, 10 μM NAD) corresponding to this band was cut from a parallel gel The gel was crushed and the protein was eluted with 50 mM buffer A containing bovine serum albumin. About 5–10 % of the applied activity was recovered in

Table 1. Purification of UDP glucose 4-epimerase from Ehrlich ascites carcinoma cells.

Steps	Total activity (units)	Total protein (mg)	Specific activity (units/mg protein)	Fold of purification	Yield (%)
Crude	7·7	3600	0·0021	1	100
1st ammonium sulphate fraction	7·7	900	0·0086	4	100
Calcium phosphate gel	6·6	300	0·0220	12	85
2nd ammonium sulphate fraction	4·3	149	0·0285	16	55
DEAE eluant (concentrated)	2·6	15	0·1710	94	33
Affinity eluant (concentrated)	0·6	0·03	23·6	11238	8

determined on a calibrated Sephadex G-200 column and was calculated to be 96,000 ±6,000. In this case the column was run at 8°C and was washed with 50 mM sodium phosphate buffer pH 7·4. Since widely different molecular weight values have been reported for the bovine liver enzyme by two different groups of workers (Geren *et al.*, 1977; Langer and Glaser, 1974), we repeated the experiment with a less purified enzyme fraction (DEAE-eluant) and under different filtration conditions. In all the cases, the molecular weight approximately corresponded to the value determined above.

Dependence on the stability of the enzyme on added NAD

Unlike epimerase from other sources, the ascites epimerase exhibited a strong dependence on added NAD for stability. During the course of purification of this enzyme, it lost its activity rapidly when kept frozen at −10°C. Elution of the enzyme from DEAE-cellulose column with increasing ionic strength of the eluting buffer also resulted in almost total loss of activity. A systemic search revealed that the enzyme could be stabilized by the specific addition of NAD. Typical stabilizing agents like 2-mercaptoethanol or EDTA or a combination of both of these failed to give any protection against this gradual inactivation. The total dependence of the enzyme for stability on the presence of exogenously added NAD could be demonstrated clearly when a partially purified enzyme fraction was passed through a series of Sephadex G-50 columns, each equilibriated and eluted with varying concentrations of NAD (table 2). The table shows that in the absence of NAD, the activity of the enzyme is rapidly lost, whereas it is effectively protected in the presence of 10 μM NAD. The inactivation was probably not due to the action of serine proteases since, addition of phenylmethylsulphonylfluoride at the early stage failed to prevent inactivation to any significant extent.

The requirement of NAD for stability of the enzyme could be further demonstrated when a heat inactivation experiment was carried out in the presence of NAD. The

Table 2. Recovery of activity from Sephadex G-50 column equilibriated with varying amounts of NAD.

Concentration of NAD (μM)	Activity (%)		
	0 h	24 h	48 h
—	25	2	0
0·5	38	25	13
2·5	90	61	40
10·0	94	90	88

For this experiment Sephadex G-50 columns (30 cm × 0·9 cm) were equilibriated with 20 mM sodium phosphate buffer pH 7·4, 1 mM 2-mercaptoethanol, 1 mM EDTA and varying concentrations of NAD as shown above. Enzyme (0·1 unit considered as 100% active) from dialyzed second ammonium sulphate fraction was passed through and eluted from each of these columns in the presence of the above eluting buffers. The eluted enzymes in the void volume were immediately assayed (considered as 0 h) and then frozen for subsequent assays.

Table 3. Protection of UDP glucose 4-epimerase against heat inactivation in the presence of varying NAD concentration

Concentration of NAD (μM)	Activity (%)		
	0 min	2 min	5 min
—	30	12	0
2·0	45	25	12
10·0	78	66	54

For this experiment concentrated enzyme fraction from DEAE cellulose column was passed through a Sephadex G-50 column equilibriated with buffer A. Aliquots were taken in tubes containing varying amounts of NAD as indicated in the table. The tube without NAD served as the control. The tubes were placed in water bath at 42°C. As soon as the solution reached the temperature of the water bath, aliquots were removed and placed in crushed ice. This served as the zero time control. Aliquots were withdrawn from each tube at different time intervals as indicated in the table and placed in the ice bath and the rate was measured by coupled assaay system as mentioned previously.

within 4–5 min at this temperature. NAD at a concentration of 10 μM could provide substantial protection against this rapid inactivation. Further increase in the concentra-

Mode of participation of NAD in catalysis

Apart from its stabilizing role for the ascites enzymes, NAD is an obligatory coenzyme for the catalytic process. Extensive mechanistic studies on UDP galactose 4-epimerase from different sources have shown, the enzyme to be an oxidoreductase and the catalysis is mediated through NAD (Gabriel *et al.*, 1975). The mode of participation of NAD, however, varies from source to source. In the case of *E. coli* and *S. fragilis* enzymes, the coenzyme is tightly bound to the apoenzyme, whereas, the enzyme from mammalian sources needs exogenous NAD for catalysis (Glaser, 1972). Employing the two step assay procedure, the catalysis in the case of the ascites enzyme was found to be totally dependent on exogenously added NAD (table 4). The control sample containing no exogenous NAD, occasionally showed some trace activity (less than 3% of the maximum activity) but this was probably due to NAD contamination that could not be removed by dialysis and gel filtration. When a large number of analogues of NAD such as NADP, acetylpyridine-NAD, thio-NAD, etheno-NAD and nicotinamide adenine mononucleotide (NMN) were tested for their capacity to replace NAD as the coenzyme, none of these excepting etheno-NAD was found to be active. Etheno-NAD has recently been shown by Samanta and Bhaduri (1983) to be fairly active in replacing NAD in a reconstituted yeast enzyme system. Interestingly, NADP failed to show even trace activity in this system.

The free nature of the coenzyme in relation to the apoenzyme was further confirmed

Table 4. Role of NAD in catalysis.

NAD/its analogues	UDPG formed (nmol)
—	0·5
NAD (1·0 μM)	4·7
NAD (5 μM)	19·4
NAD (10 μM)	19·1
Etheno NAD (10 μM)	1·9
Etheno NAD (25 μM)	2·1
α-NAD (25 μM)	0·4
NADP (25 μM)	0·2
Acetyl pyridine NAD (25 μM)	0·5
De-amino NAD (25 μM)	0·6
NMN (25 μM)	0·7

The incubation mixture (1 ml) contained 100 μmol of glycylglycine buffer, pH 8·4, 0·5 μmol of UDP galactose and different concentrations of NAD and its analogues as indicated in the table. Reactions were started by the addition of 0·004 unit of enzyme in each case and terminated with chloroform after 5 min. This enzyme fraction obtained from the DEAE-cellulose column was earlier freed of NAD by passing through a by the 'reductive inhibition' test for this epimerase. Kalckar and his group had shown that for *E. coli* or yeast epimerase that have tightly bound NAD at the catalytic site, the coenzyme can be slowly but irreversibly reduced to NADH in the presence of UMP and high concentration of a suitable sugar (Gabriel *et al.*, 1975). The slow conversion of NAD to NADH on the enzyme surface leads to inactivation of the enzyme and is a highly characteristic process for only to those epimerase that have the coenzyme tightly bound to the enzyme surface (Gabriel *et al.*, 1975; Pal and Bhaduri, 1971). When the purified ascites enzyme was exposed to high concentration of glucose in the presence of UMP, the enzyme retained almost total activity even after prolonged incubation. The control yeast enzyme, on the other hand, was completely inactivated in 6 h (figure 1). The reason for a slight but consistent loss of activity for the ascites enzyme in the first 1 h is not clear at the moment. The exogenous NAD that is needed for catalysis was found to have a strong affinity for the apoenzyme and the K_m was determined to be 2·5 μM at pH 8·4 by the two step assay procedure.

Determination of kinetic parameters

The ascites epimerase was found to be active over a wide range of pH. Using glycylglycine buffer, the optimum pH of the enzyme was found to be 8·4. For this

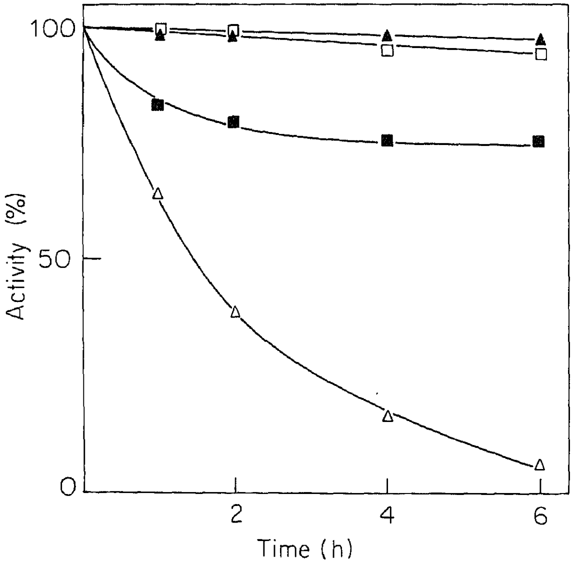

Figure 1. Test for reductive inactivation for ascites epimerase. For this experiment, 0·1 unit of ascites enzyme, obtained after DEAE-cellulose column purification step, was incubated in a total volume of 0·1 ml, containing 10 μmol of phosphate buffer pH 7·4, 0·1 μmol of UMP and 1 μmol of glucose. An aliquot (1 μl) was taken out at indicated intervals and the activity of the enzyme was assayed by coupled assay method. The progress of the reaction is, experimental (■); control tube (□) *i.e.* minus glucose. As separate controls, purified *S. fragilis* enzyme (0·1

determination, the two step assay had to be employed since UDP glucose dehydrogenase, the coupling enzyme, showed significantly lower activity near neutral pH. The two step assay was also employed for the determination of K_m for the substrates. K_m for UDP galactose and UDP glucose were found to be 19 μM and 800 μM respectively. The equilibrium value for the reaction at pH 8·8 was found to be 2·7 ± 0·3 (UDP glucose to UDP galactose). The slightly lower observed value, compared to other published values for epimerase from other sources might be due to differing conditions of pH and temperature at which equilibrium was attained.

Effect of related metabolites on catalytic activity of the enzyme

Since any drastic modulation of the activity of epimerase by metabolites related directly or indirectly to galactose metabolism may have a significant effect on UDP galactose pool, a large number of these compounds were tested for their potential effect on epimerase activity. These included nucleoside mono-, di- and triphosphates; sugars and sugar phosphates; high energy compounds and nucleotide sugars.

Only UMP and NADH had significant inhibitory effect on the ascites enzyme. The enzyme was strongly and competitively inhibited by UMP and the K_i was calculated to be 40 μM. UDP had some inhibitory effect but its binding affinity for the substrate site was much lower than that of UMP. NADH was found to be a very potent inhibitor for the ascites enzymes. Interestingly, the extent of inhibition depended significantly on the pH of the medium (figure 2). Thus, while the enzyme activity is inhibited by only 25 % at pH 8·8 at a NADH to NAD ratio of 0·16, the same relative concentration of the reduced to oxidized form of the coenzyme, resulted in inhibition by 67 % at pH 7·6. The pronounced effect of the pH of the medium can also be seen from the fact that to achieve the same extent of inhibition, nearly four times NADH is needed at pH 8·8 than at pH 7·6. Working with epimerase from crude HeLa cell extracts, Kalckar and his group were the first to note the pronounced effect of pH on the extent of inhibition by NADH (Robinson *et al.*, 1966). Kinetic analysis of the inhibition process further demonstrated that NADH is a strict competitor of NAD for its binding site (figure 3). The K_i of NADH was calculated to be 0·11 μM indicating that it had a much stronger binding affinity for the pyridine nucleotide binding site of the enzyme than NAD itself.

Discussion

This report presents for the first time a detailed study of UDP glucose 4-epimerase from a tumor source. The general characteristics of the enzyme from the ascites cells is broadly similar to that found for the enzyme from other mammalian sources that include the calf-liver enzyme (Geren *et al.*, 1977; Langer and Glaser, 1974) and the mammary gland enzyme (Geren *et al.*, 1977). The molecular weight of 96,000 determined for the ascites enzyme corresponds more closely to the value of 80,000 reported for the calf-liver enzyme by Langer and Glaser (1974) and not to 39,000 as

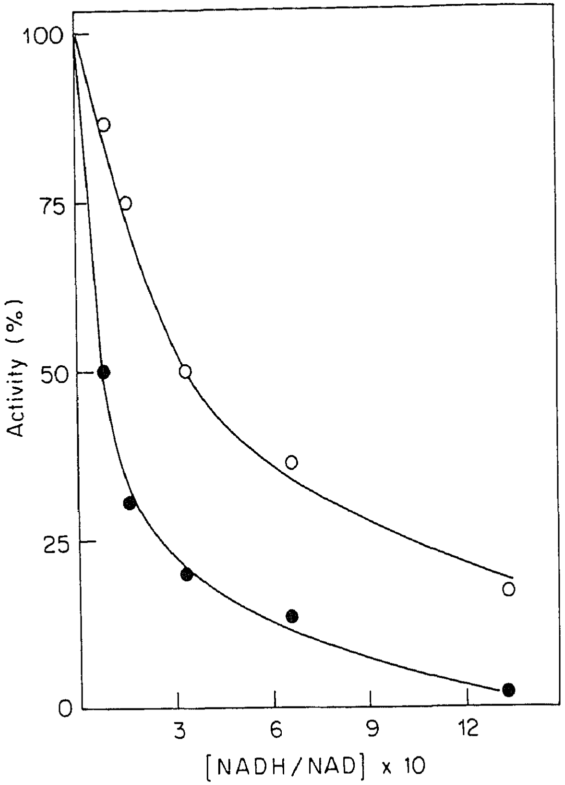

Figure 2. Inhibition of ascites epimerase by NADH at different pH values. The enzyme used for this experiment was a DEAE-cellulose eluant that was freed of added NAD by passing through a Sephadex G-50 column. Each incubation tube contained a total volume of 1 ml, 100 μmol of glycylglycine buffer either at pH 7·6 or at pH 8·8, 0·002 units of the enzyme, 0·005 μmol of NAD and varying concentrations of NADH. Reactions were initiated by addition of UDP galactose and carried out for 6 min. Formation of UDP glucose was measured by usual procedures. (●) and (O) indicate extent of inhibition at pH 7·6 and 8·8 respectively.

exogenous NAD for stability. Neither the purified calf-liver enzyme nor the mammary enzyme shows such absolute dependence on NAD for stability (Glaser, 1972). Interestingly, all reports on the crude extract of epimerase from various transformed tumor sources show that the stability of the enzyme is enormously enhanced in the presence of NAD (Mitchell *et al.*, 1975; Chacko *et al.*, 1972). Mitchell *et al.* (1975) had shown that lymphoblast cultures derived from leukocytes of individuals lacking in epimerase showed significant levels of renewed epimerase activity that became stable only in the presence of NAD. The distinguishing property of the tumor epimerase along with the fact that the epimerase level becomes several fold higher in Morris hepatoma cells (Bauer *et al.*, 1980), raises the interesting possibility that a new isozymic form of the

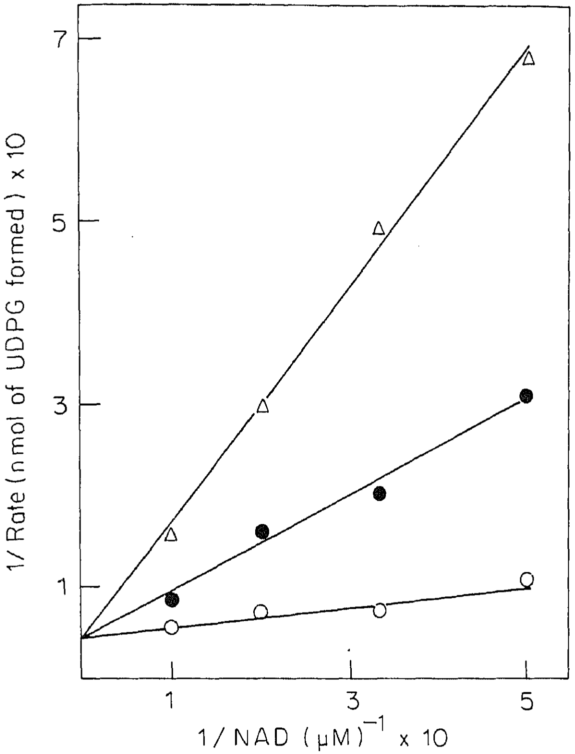

Figure 3. Competitive inhibition of epimerase activity by NADH. All the assays for this experiment were carried out by two-step assay procedure. (O), (●) and (Δ) are in the absence of NADH, in the presence of 0·5 μm NADH and 1·0 μM NADH, respectively.

Our work with pure ascites enzyme shows that extreme sensitivity to NADH of the tumor enzyme at cellular pH may have a profound effect on the formation of UDP galactose pool for these cells. Similar conclusions were reached by Robinson *et al.* (1966) from their work with crude extracts of HeLa cells. However, in the absence of any uniform indication that the NADH to NAD ratio goes up in transformed cells (Aisenberg, 1961), the significance of such an inhibition of epimerase on the synthesis of cell surface glycoconjugates remains speculative at the moment.

Acknowledgement

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Biochemical analysis of galactose induced bacteriostasis in *galT* mutants of *Escherichia coli* K 12

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Abstract. *Escherichia coli* mutants completely defective in galactose-1-phosphate uridyl transferase (EC 2.7.7.10) and growing in glycerol medium undergo rapid cessation of growth when exposed to galactose. Toxicity due to galactose is equally pronounced when glycerol is replaced by other carbon sources, like succinate and proline. Gas chromatographic analysis failed to detect even trace amounts of galactitol. Moreover, galactose-1-phosphate had no inhibitory role on some of the critical enzymes of cellular metabolism. General loss of energy (ATP) due to futile phosphorylation of galactose is probably the cause of bacteriostasis. The *galT* mutants can serve as models of human transferaseless galactosemia only to a limited extent.

Keywords. Galactose toxicity; galactitol; *galT* mutants; *Escherichia coli.*

Introduction

Galactose is catabolized in a wide variety of living systems including most microorganisms by sequential action of three specific enzymes of Leloir pathway. The enzymes galactokinase (EC 2.7.1.6), galactose-1-phosphate uridyl transferase (EC 2.7.7.10) and uridine diphosphate galactose-4-epimerase (EC 5.1.3.2) abbreviated as kinase, transferase and epimerase act in the following way:

$$\text{Galactose} + \text{ATP} \xrightarrow{\text{kinase}} \text{Galactose-1-P} + \text{ADP} \tag{1}$$

$$\text{Galactose-1-P} + \text{UDPG} \xrightleftharpoons{\text{transferase}} \text{UDPgalactose} + \text{G-1-P} \tag{2}$$

$$\text{UDPgalactose} \xrightleftharpoons{\text{epimerase}} \text{UDPglucose} \tag{3}$$

$$\text{Sum: Galactose} + \text{ATP} \xrightarrow{\qquad\qquad} \text{G-1-P} + \text{ADP}$$

Galactosemia, a classical hereditary disease of children, was originally shown to be due to a genetic defect in the expression of the transferase enzyme (Isselbacher *et al.*, 1956; Isselbacher, 1960). Galactose-1-phosphate that accumulated in various tissues was suspected to be the immediate causative factor for the development of diverse pathological symptoms that manifest in the diseased state. Recent clinical and biochemical research has, however, clearly shown that galactosemia comprises of at least two distinct genetic diseases, galactose-1-phosphate uridyl transferase deficiency galactosemia and galactokinase deficiency galactosemia (Segal, 1983). In both cases, large quantities of galactitol, the reduced product of galactose, accumulates and interfers with normal cellular functions by changing osmomolarity and inositol metabolism (Wells *et al.*, 1965; Kinoshita, 1969; Knull *et al.*, 1974; Segal, 1983).

Consequent to the original discovery of the enzymatic basis of galactosemia, *galT* mutants of *Escherichia coli* were shown to undergo bacteriostasis in the presence of galactose with glycerol as the carbon source and large quantities of galactose-1-phosphate accumulated during the process (Yarmolinsky *et al.*, 1959; Kalckar *et al.*, 1959; Sundarajan *et al.*, 1962). Galactose-1-phosphate was regarded as the toxic agent that disrupted normal metabolism and *galT* mutant was implied as the model for human galactosemic cells (Kalckar *et al.*, 1959; Sundarajan *et al.*, 1962). In view of the recent findings regarding the metabolic basis of galactose toxicity in humans and in experimental animals (Segal, 1983), and also because of its intrinsic interest, we decided to reinvestigate the problem of galactose toxicity in *galT* mutants of *E. coli*. Our analysis shows that galactitol is not an accumulated product during bacteriostasis. Further galactose-1-phosphate probably has no role in developing bacterial toxicity and the bacteriostasis can be adequately explained by an irreversible loss of cellular energy.

Materials and methods

Bacterial strains

All strains of bacteria utilized in the experiments were derivatives of *E. coli* K 12 and were generously donated by B. J. Bachmann, Curator of *E. coli* Genetic Stock Centre of

Table 1. Bacterial strains.

Strain	Phenotype[a]	Genotype and characteristic[b]	Origin
CA1	Gal^+	Hfr, λ^-, isogenic parent of CA190, CA13	S. Brenner
CA190	Gal^-	Hfr, λ^-, *galK*35	S. Brenner
CA13	Gal^-	Hfr, λ^-, *galT*118	S. Brenner
W3110	Gal^+	F^-, λ^-, isogenic parent of W3092, W3104	Lederberg
W3092	Gal^-	F^-, λ^-, *galK*2	M. L. Morse
W3104	Gal^-	F^-, λ^-, *galT*12	M. L. Morse

ither on M9 or 7.0P minimal medium (Miller, 1972; Buettner *et al.*, 1973). Unless otherwise mentioned, glycerol (0·2 %) was the carbon source in the minimal medium. 0 μg of thiamine hydrochloride per ml was used when required.

Biochemicals

Galactose, free from glucose was obtained from Sigma Chemical Co., St. Louis, Missouri, USA. All other biochemicals and enzymes were also obtained from the same ource. Other reagents that were locally obtained were of analytical grade.

Enzyme assays and metabolite estimation

n cell-free extracts, galactokinase was assayed by the two-step assay procedure of Soffer (1961). Galactose-1-phosphate uridyl transferase was assayed by coupling the eaction with phosphoglucomutase and glucose-6-phosphate dehydrogenase system. The assay was based on the earlier published method (Kalckar *et al.*, 1959). UDPglucose-4-epimerase was assayed by either coupling directly with UDPglucose dehydrogenase system or with the same enzyme but by a two-step assay procedure. These methods are essentially the same as described for the yeast enzyme by Ray and Bhaduri (1975). The cell-free extracts for all these assays were prepared by grinding the washed cell-pellet with twice the weight of glass powder and then extracting with ·05 M Tris-HCl buffer pH 7·4 containing 1 mM EDTA and 2 mM 2-mercaptothanol. Broken cells were precipitated by centrifugation at 20,000 *g* for 15 min and the lear supernatant utilized for enzyme assay.

For estimation of ATP, UDPglucose and UDPgalactose, cells were harvested and washed rapidly and then extracted with 10 % cold perchloric acid. After centrifugation he clear supernatant was neutralized to pH 6·0–6·5 with 5 M K_2CO_3. The precipitated potassium salts were removed after 1 h and the clear supernatant was ready for the stimation of the metabolites by enzymatic methods. ATP was estimated by hexokinase-glucose-6-phosphate dehydrogenase system, UDPglucose was estimated by UDPglucose dehydrogenase system and UDPgalactose was estimated by first xhausting UDPglucose and then adding the epimerase in the system. The methods were essentially based on published procedures (Bergmeyer, 1974). Faster processing of he cells by direct transfer to perchloric acid and subsequent assay by luciferase system gives a somewhat higher and possibly more accurate value for ATP in *E. coli* (Cole *et al.*, 1967). Our procedure which is essentially an adaptation of an earlier method of Franzen and Binkley for *E. coli* (Franzen and Binkley, 1961) was found to be adequate o demonstrate the trend in the fluctuation of ATP content under varying physiological conditions.

For the estimation of galactose-1-phosphate, the cell-free extract obtained by grinding with glass powder was placed in boiling water bath for 2 min. After removal of he precipitated proteins, a suitable aliquot was added to 1 ml of the solution containing 100 μmol of UDPglucose, 0·5 μmol of NADP, 0·01 μmol of glucose-1-6-diphosphate along with 0·04 unit of phosphoglucomutase, 0·04 unit of glucose-6-

phosphate dehydrogenase and 0·02 unit of purified yeast galactose-1-phosphate uri transferase. Increase in absorbancy at 340 nm due to the formation of NADPH wa direct measure for galactose-1-phosphate in the aliquot. All the estimation procedu for the metabolites were standardized with suitable internal controls and the erro estimation did not exceed ±20%.

Detection of galactitol in cell free extract

Two g of *galT* cells (wet wt) harvested under conditions of bacteriostasis were disrup with glass powder and twice extracted with 0·05 M Tris-HCl buffer pH 7·4. The cl supernatant obtained after centrifugation was deproteinized by heating at 90°C 10 min. After centrifugation, the clear supernatant was divided into two parts. In part 2 μg of galactitol was added as an internal control. To both the samples IR- (OH^-) and IR-120 (H^+) resins were added batchwise until the pH corresponded to pH of distilled water. Processed samples were next evaporated to dryness and t acetylated with acetic anhydride and pyridine solution (Sawardeker *et al.*, 1965). liquid chromatographic analysis was carried out in a Hewlett-Packard model 573 gas chromatograph with a FID detector.

Results

Effect of galactose on growth

Figure 1 shows the dramatic effect of added galactose (10 mM) on the exponenti growing cells (W3104) defective in the transferase. The inset shows the results in te of viable cells. Obviously, a strictly bacteriostatic phenomenon is observed in this c Addition of even much lower concentration of galactose (0·5 mM) showed a sim effect. The results further show that the addition of glucose (10 mM) promptly relie the toxicity and growth ensued in a normal manner. Galactose failed to show significant toxic effect on the *galK* mutant of this series W3092. Similar bacteriost phenomenon was observed for strains CA13 and W3107, both of which are *g* mutants.

Absence of galactitol accumulation during bacteriostasis

In order to find out if galactitol has any potential role in the development of toxicity subjected the cells undergoing bacteriostasis to gas liquid chromatographic analysis the detection of galactitol. The deionized samples, as obtained by the proced described under 'Materials and methods' section, were evaporated to dryness and t subjected to gas liquid chromatographic analysis (Sawardeker *et al.*, 1965). The res

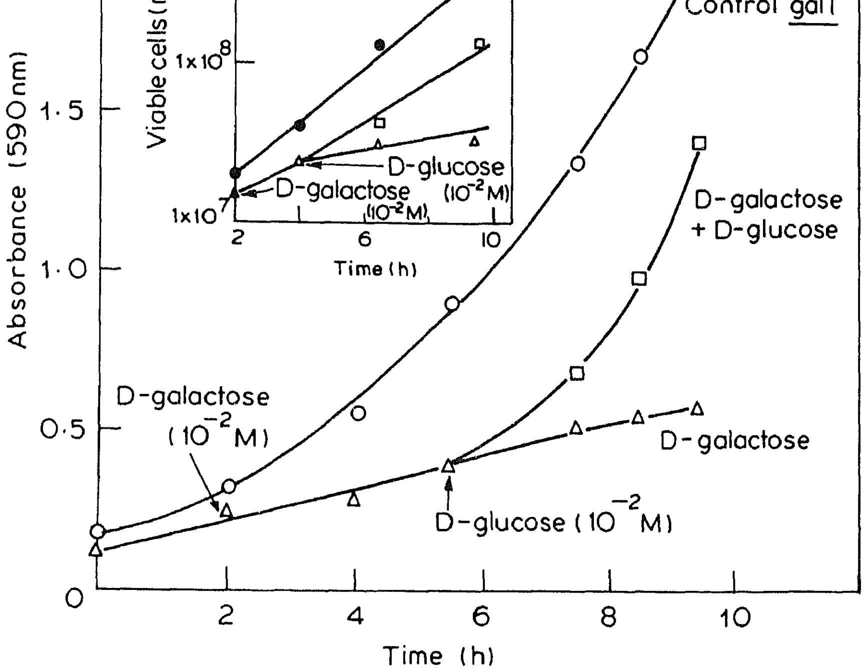

Figure 1. Effect of D-glucose on D-galactose induced bacteriostasis. A culture of W3104 in medium M9 with glycerol as carbon source was started (shake culture, 37°). After 2 h from start, galactose (10 mM) was added. The cells were incubated further till growth rate slowed down effectively and the culture was divided into two parts. To the first culture D-glucose (10 mM) was added (□) and the second culture was left unaltered (Δ). Control flask contained M9-glycerol grown culture only (O).

The inset shows viability of same *galT* mutant strain W3104 during galactose mediated inhibition of growth and glucose reversed situation. Viable cells calculated from medium values of duplicate plate counts on Luria agar plates.

Replacement of glycerol by other carbon sources

When glycerol was replaced by other carbon sources, such as, succinate (figure 3) or proline (data not shown) addition of galactose to the growing *galT* mutants resulted in essentially similar type of bacteriostatic action. Obviously, the toxic effect due to added galactose, is independent of the nature of the carbon source and of the dominant route of catabolism.

Metabolite analysis during bacteriostasis

Table 2 presents the concentration of some key metabolites of galactose metabolism under conditions of normal growth, bacteriostasis and reversal of growth stasis with glucose. This is also compared with the values obtained with the wild type cells under

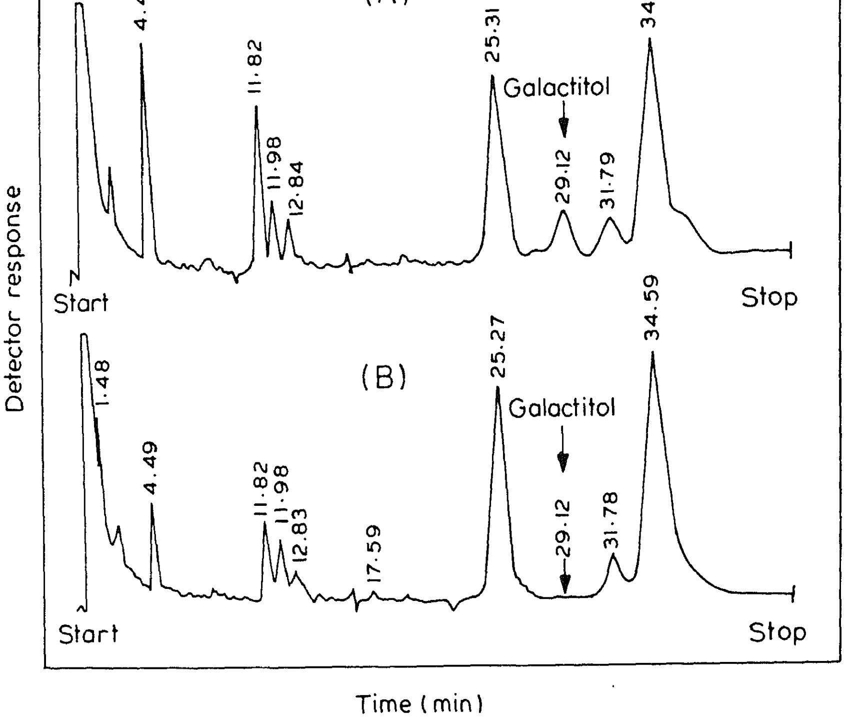

Figure 2. Gas liquid chromatogram for estimation of galactitol in cell free extract of galactose induced bacteriostatic cells of *galT* mutant strain of *E. coli* K 12. Gas liquid chromatography experiments were carried out on a Hewlett-Packard model 5730 A gas chromatograph with FID detector. Glass column (1·83 m × 6 mm) containing 3 % ECNSS-M on gas chrom Q (100–200 Mesh) at 290°C was used. A reporting integrator 3390 (H. P.) was used to record retention times and molecular ratios.

A. Sample containing known galactitol as internal control.

B. Sample containing D-galactose treated *galT* cell free extract.

The peak corresponding to retention time 29·12 min was identified as galactitol.

similar growth conditions. Clearly, growth retardation was accompanied by a significant fall in the level of ATP and an equally significant rise in the level of galactose-1-phosphate. Interestingly, concentrations of both UDPgalactose and UDPglucose were also lowered, though their relative concentrations remained essentially unchanged. Reversal with glucose resulted in a trend towards normalcy for all these metabolites. Possible inhibitory effect of galactose-1-phosphate was also tested in *E. coli* glycerokinase, phosphoglucomutase, UDPglucose pyrophosphorylase and glucose-6-phosphate dehydrogenase. Galactose-1-phosphate was reported to have some inhibitory effect on these enzymes (Lermain, 1959; Sidbury, 1960; Sundarajan, 1963). We could not demonstrate any inhibitory effect of the metabolite on the activity of any of these enzymes excepting a moderate one on glycerokinase (data not shown).

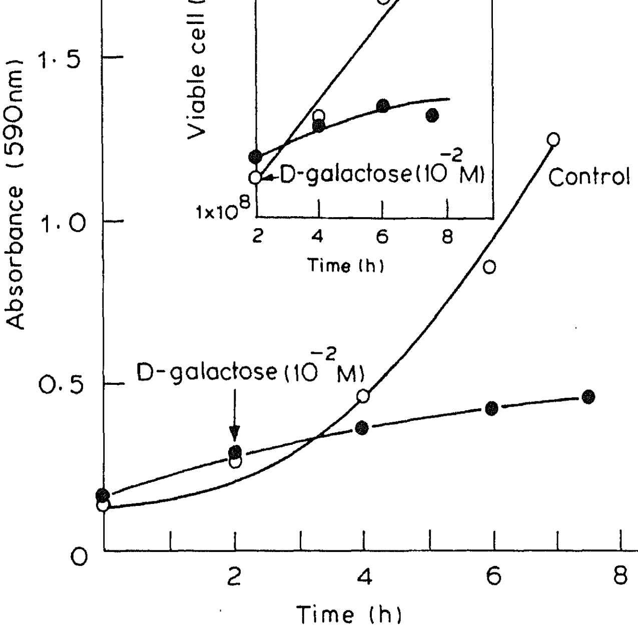

Figure 3. Galactose induced bacteriostasis of *E. coli* W3104 (*galT*) with succinate as carbon source. Cells were grown in shake culture at 37° in medium 7·0P containing 20 mM succinate as carbon source. Galactose at a final concentration of 10 mM added at the arrow. (O), 7·0P—succinate; (●), 7·0P—succinate + 10 mM galactose.

The inset shows the same result in terms of viability of cells.

nzyme make-up under conditions of bacteriostasis and its reversal

Vhen the levels of the enzymes of galactose pathway were measured under various rowth conditions for both the wild type and the *galT* mutants, a clear correlation etween bacteriostasis situation and the level of galactokinase became apparent. resence of glucose repressed the synthesis of the enzyme though the level of enzyme vas higher than in the uninduced situation (table 3).

iscussion

ur studies with the *galT* mutants clearly demonstrated the limitations of these nutants as models of human transferaseless galactosemia. In this case, galactitol is not a lead-end product of metabolism (figure 2) and therefore, cannot play any role in the levelopment of toxicity. Growth experiments in the presence of galactitol also leads to he same conclusion. As expected galactose-1-phosphate accumulates to a great extent

Table 2. Quantitative analysis of the metabolites of D-galactose in galactose induced bacteriostatic condition of *galT* mutants and on reversal of stasis with glucose[a].

Relevant characteristic	Growth condition at 37°C	D-galactose-1-phosphate (μmol/g dry wt. of cell)	ATP (μmol/g dry weight of cell)	UDPglucose (μmol/g dry wt. of cell)	UDPgalactos (μmol/g dry w of cell)
Wild type	M9-glycerol	0·30	2·32	1·18	0·26
	M9-glycerol + galactose (10 mM)	0·45	2·58	0·87	0·14
	M9-glycerol + galactose (10 mM) + glucose (10 mM)	0·33	1·80	0·86	0·16
galT	M9-glycerol	0·40	1·86	0·85	0·21
	M9-glycerol + galactose (10 mM)	3·20	0·34	0·034	<0·01
	M9-glycerol + galactose (10 mM) + glucose (10 mM)	0·60	1·26	0·61	0·13

[a] Results are average of three separate set of experiments.

Table 3. Activities of enzymes of galactose metabolic pathway in a *galT* mutant under conditions of stasis and its reversal.

Strain designation and relevant characteristic	Growth condition (37°C)	Specific activity		
		Kinase[a]	Transferase[b]	Epimerase[c]
CA1, Wild type	Glycerol	0·12	0·48	2·1
	Galactose (10 mM)	0·78	5·12	13·9
	Galactose (10 mM) + glucose (10 mM)	0·26	1·68	4·03
CA13, *galT*	Glycerol	0·092	< 0·01	1·82
	D-galactose (10 mM)	0·68	< 0·01	11·69
	D-galactose (10 mM) + glucose (10 mM)	0·18	< 0·01	4·51

[a] Expressed as μmol of galactose-1-phosphate formed/h/mg protein.

[b] Expressed as μmol of substrate converted/h/mg protein.

[c] Expressed as μmol of UDPglucose formed/h/mg protein.

(table 2) but its critical role in the development of bacteriostasis is questionable. If the compound was inhibiting any specific enzyme of the glycolytic pathway, bacteriostasis was not expected to be observed with succinate or proline as sole carbon sources (figure 3), since these compounds are catabolized by entirely different channels before they get into the citric acid cycle. Growth experiments with these compounds also demonstrate that the repression of glycerokinase synthesis in the presence of galactose (Sundarajan, 1963) is not an adequate explanation for the development of toxicity. Galactose-1-phosphate does not seem to have any direct role in decreasing the nucleotide pool (table 2) as neither UDPglucose pyrophosphorylase nor UDPglucose-4-epimerase from *E. coli* was inhibited by this metabolite. A more likely cause for the cessation of growth is unilateral loss of metabolic energy or ATP due to futile phosphorylation of galactose. Similar energy or phosphate 'trap' was earlier indicated for 2-deoxy-D-galactose toxicity in experimental animals by Keppler and his group (Starling and Keppler, 1977; Lattke *et al.*, 1979). The reversal of bacteriostasis with glucose is possibly due to the metabolic facility of this sugar as the preferred carbon source. However, repression of the galactokinase (table 3) may also contribute significantly in releiving the energy 'trap'. The lowering of the nucleotide pool may simply reflect a general dislocation in all biosynthetic processes due to loss of ATP. It would be interesting to investigate the problem further with 2-deoxy-D-galactose as a non-metabolizable sugar analogue of galactose.

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Product stabilization of the two forms of carboxypeptidase A from buffalo pancreas

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Abstract. The two forms of buffalo carboxypeptidase A lose a part of their activity when incubated at their optimal temperature. Both the forms are protected from heat denaturation by L-phenylalanine, one of the reaction products while the other reaction product hippuric acid provides only limited protection. It has also been shown that L-phenylalanine and hippuric acid bind at the same site of the enzyme and that the release of L-phenylalanine follows that of hippuric acid. On this basis, a new mechanism for the hydrolysis of acyldipeptides by carboxypeptidase A has been proposed.

Keywords. Product L-phenylalanine; stabilization; carboxypeptidase A.

Introduction

Stabilisation of the enzyme against denaturation by substrates or cosubstrates has been reported in a number of cases. Glucose-6-phosphate isomerase from *Bacillus stearothermophillus* (Muramatsu and Nosoh, 1971), dihydrofolate reductase from *Rhizoctonia solani* (Reddy *et al.*, 1976) and adenosine triphosphatase (Pullman *et al.*, 1960) have been reported to be protected against thermal inactivation by the presence of their substrates: glucose-6-phosphate, dihydrofolate and adenosine triphosphate respectively. The two forms of carboxypeptidase A from buffalo pancreas designated as $CPAb_1$, and $CPAb_2$ have a high optimum temperature of 50°C (bovine carboxypeptidase A usually being assayed at 25°C) and lost a part of the activity when incubated alone at their optimum temperature. The product stabilisation of goat carboxypeptidase B, for the first time has been reported by Dua and Srivastava (1981) which raised an interesting question whether this phenomenon is common to all types of carboxypeptidases, irrespective of their source. So it was decided to study the thermal stability of the two forms of carboxypeptidase A and to investigate the effect of the presence of the substrate and the reaction product(s) on heat denaturation of $CPAb_1$ and $CPAb_2$ in detail.

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of the chemicals were from M/s British Drug House, UK or from E. Merck, Germany and were of analytical grade, unless stated otherwise.

The two forms of $CPAb_1$ and $CPAb_2$ were purified according to the procedure of Sud and Dua (1984a). Carboxypeptidase A activity was assayed by incubating 0·1 ml aliquot of the enzyme solution with 0·6 ml of 0·025 M concentration of substrate (NCbz-glycyl-L-phenylalanine or hippuryl-L-phenylalanine) in 0·02 M Tris-0·1 M NaCl buffer, pH 7·5, and by estimating the released phenylalanine in 15 min at 50°C with ninhydrin by measuring its absorbance at 565 nm. The activity is expressed in enzyme units, 1 enzyme unit being equal to 1 μmol of amino acid liberated/min under the assay conditions. Protein concentration was determined either by the method of Lowry *et al.* (1951) or spectrophotometrically by the method of Warburg and Christian (1941).

Results and discussion

Thermal stability of $CPAb_1$ and $CPAb_2$

The optimum temperature of the two forms, $CPAb_1$ and $CPAb_2$ for the hydrolysis of NCbz-glycyl-L-phenylalanine was determined (figure 1). Both forms show maximal

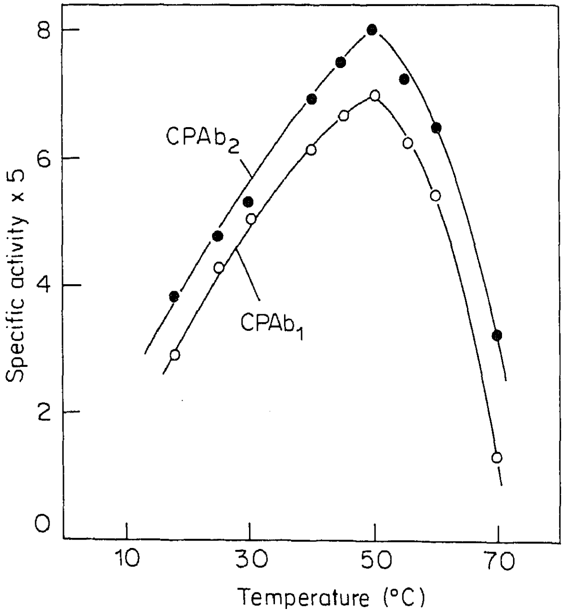


Figure 1. Effects of Temperature on the activity of $CPAb_1$ and $CPAb_2$.
Aliquots (0·1 ml) of protein solution (0·1 mg/ml in 0·3 M sodium acetate buffer, pH 7·0) were assayed for carboxypeptidase A activity at different temperatures using NCbz-glycyl-L-phenylalanine as substrate.

rates of hydrolysis at 50°C. This was surprising as the known bovine carboxypeptidase A has a much lower optimum temperature.

In general, inactivation of enzymes in solution increases rapidly with rise in temperature. So the thermal stability of both the forms was checked by heating the protein solution at different temperatures for 10 min, cooling, and then assaying their activity (figure 2). It is evident that both forms of the enzyme start loosing activity when incubated at higher temperatures, $CPAb_1$ being slightly more stable than $CPAb_2$ which confirms earlier the report of Sud and Dua (1984b) on half life studies at 50°C on the two forms. Also as $CPAb_1$ has a longer polypeptide chain than $CPAb_2$ (Sud and Dua, 1984a) it supports Petra and Neurath's (1969) view that lengthening the polypeptide chain confers greater stability.

The thermal stability curve shows that $CPAb_1$ and $CPAb_2$ lose 20% and 35% of their activity when incubated at their optimum temperature of 50° for 10 min, suggesting that the enzymes are being protected against thermal denaturation by the substrate and/or either one or both the reaction products. It was, therefore, decided to determine the heat denaturation effects in the presence of substrate and reaction product(s) separately (table 1).

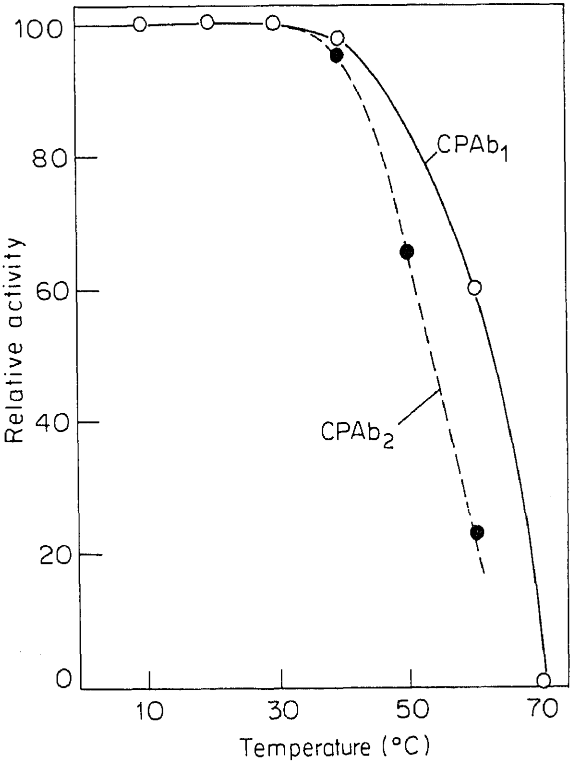


Table 1. Effect of substrate/product(s) and varying molar ratio of products on thermal stability of $CPAb_1$ and $CPAb_2$.

Preincubated with	Treatment	% Activity left $CPAb_1$	 $CPAb_2$
Control	Untreated	100·00	100·0
Control	Heated	12·0	5·9
L-Phenylalanine	Heated	85·3	80·4
Hippuric acid	Heated	18·5	20·0
Hippuryl-L-Phenylalanine	Heated	42·3	30·8
Hippuric acid: L-Phenylalanine			
Molar ratio 1:1	Heated	55·2	54·0
2:1	Heated	32·4	30·8
3:1	Heated	18·8	19·2
4:1	Heated	10·2	12·0

Aliquots (2 ml) of each protein solution (0·12 mg protein/ml in 0·3 M sodium acetate, pH 7·0) were incubated in the presence of 0·025 M product/substrate (in 0·005 M Tris-acetate-0·1 M NaCl, pH 7·5) or hippuric acid: L-phenylalanine in given ratios (L-phenylalanine being added 30 min after the addition of hippuric acid at room temperature) at room temperature for 30 min and then heated at 60°C for 10 min, ice-cooled, dialysed against 0·005 M Tris acetate buffer-0.1 M NaCl, pH 7·5, and were assayed for carboxypeptidase A activity using hippury-L-phenylalanine as substrate. Control stands for native enzyme.

Stabilisation against heat denaturation.

Effect of substrate and reaction product(s) on heat denaturation of $CPAb_1$ and $CPAb_2$: It is evident from the data that in the presence of L-phenylalanine, one of the reaction products, $CPAb_1$ and $CPAb_2$ retain 80–85% activity as against 5–12% in its absence and 31–42% in the presence of the substrate, hippuryl-L-phenylalanine. The other reaction product, hippuric acid, provides only limited protection to both the forms of carboxypeptidase A, suggesting that the actual protection against thermal inactivation is probably provided by L-phenylalanine.

Product stabilisation was further checked by investigating the stabilisation of the enzyme protein in the presence of different concentrations of L-phenylalanine (figure 3). It is observed that stabilisation is proportional to the L-phenylalanine concentration upto 0·03 M after which the protective effect starts falling off. This led to the view that L-phenylalanine stabilises the enzyme by binding at the active site. However, at a higher concentration, phenylalanine binds probably nonspecifically at another site, and thereby disturbs the stable conformation of the protein. Similar studies with hippuric acid showed only marginal stabilisation, suggesting that hippuric acid, if it binds at the activie site at all, binds very poorly, *i.e.*, it has a comparatively higher dissociation constant than L-phenylalanine and hence is a poor stabilizer of the protein.

Effect of the order of incubation of L-phenylalanine and hippuric acid

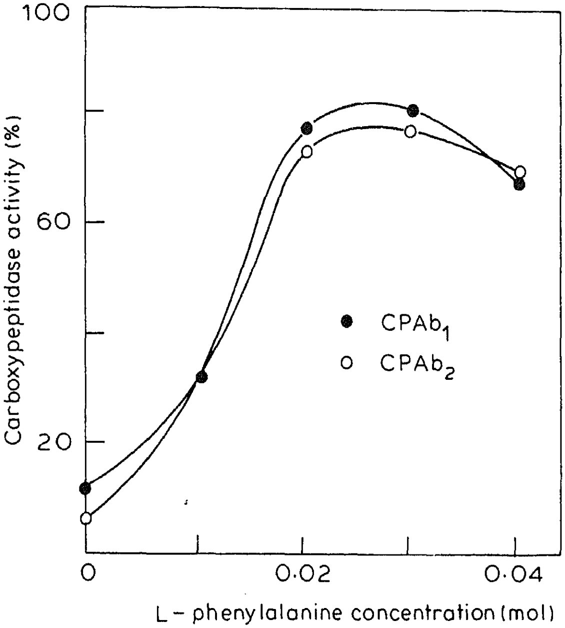


Figure 3. Effect of varying concentration of L-phenylalanine on thermal stability of CPAb$_1$ and CPAb$_2$.

Aliquots (2 ml) of each protein solution (0·15 mg protein/ml in 0·3 M sodium acetate buffer, pH 7·0) were incubated with varying concentrations of L-phenylalanine (in 0·005 M Tris acetate buffer − 0·1 M NaCl, pH 7·5) at room temperature for 30 min and then heated at 60°C for 10 min, ice-cooled, dialysed against 0·005 M Tris acetate buffer − 0·1 M NaCl, pH 7·5, centrifuged and assayed for carboxypeptidase A activity using hippuryl-L-phenylalanine as substrate. Control had no addition.

ippuric acid should, depending upon its dissociation constant, interfere to some extent ith this stability as the two products will compete for the same site. To check this, nzyme protein was incubated with optimal L-phenylalanine concentrations for 30 min efore hippuric acid was added and vice-versa. The aliquots removed after 2, 5, 10, 15 nd 30 min of second addition were heated at 60°C for 10 min, ice cooled, dialysed and ssayed for peptidase activity (figure 4A, B). It is evident that the enzyme preincubated ith hippuric acid retains only 15–18 % of the original activity when heated at 60°C for 0 min as against 6–10 % in its absence. However, addition of L-phenylalanine and icubation leads to increased stability with maximum retention of 55–58 % of original ctivity (figure 4b) compared to 80–85 % obtained in the absence of hippuric acid but in he presence of L-phenylalanine, thus indicating that there is competition between L-henylalanine and hippuric acid for binding at the active site, and as L-phenylalanine inds more strongly (K_s for L-phenylalanine is lower), at equilibrium the thermal tability attained by the enzyme is close to that achieved in the presence of L-

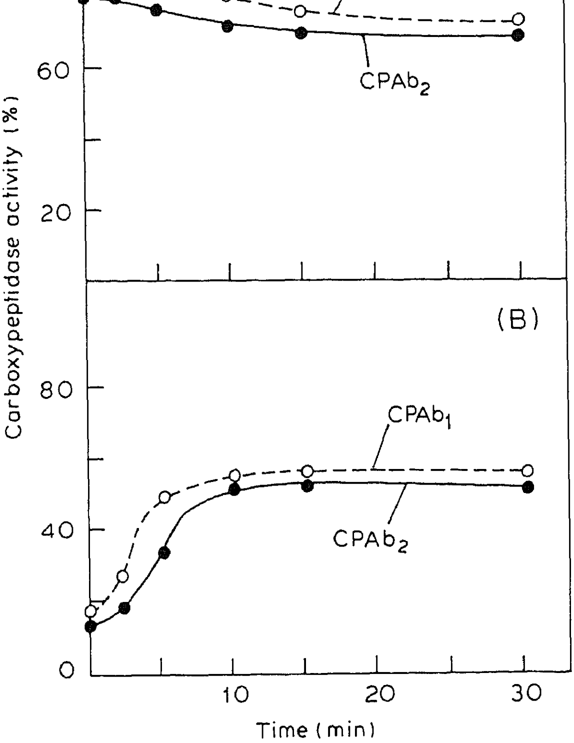


Figure 4. Effect of the order of incubation of products in the incubation mixture on heat inactivation of $CPAb_1$ and $CPAb_2$.

Aliquots (2 ml) of each protein solution (0·15 mg/ml in 0·3 M sodium acetate buffer, pH 7·0) were incubated with the optimal concentration of first additions (final concentration of 0·03 M in 0·005 M Tris acetate – 0·1 M NaCl, PH 7·5, for L-phenylalanine and 0·05 M in 0·005 M Tris acetate – 0·1 M NaCl, pH 7·5, for hippuric acid) for 30 min at room temperature followed by optimal concentration of the second additions. The aliquots were drawn after 2, 5, 10, 15 and 30 min, heated at 60°C for 10 min, ice-cooled, dialysed against 0·005 M Tris acetate buffer – 0·1 M NaCl, pH 7·5, and were assayed for carboxypeptidase A activity using hippuryl-L-phenylalanine as substrate.

A. L-phenylalanine followed by hippuric acid.

B. Hippuric acid followed by L-phenylalanine.

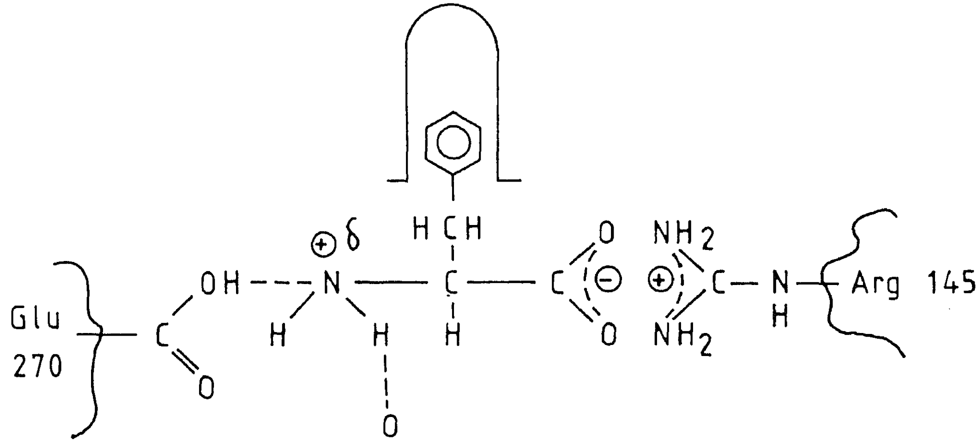
retains 80–85 % of the activity on heating at 60°C for 10 min (figure 4A). The addition of hippuric acid lowers this stability and when equilibrium is reached, within 15 min, again 65–75 % retention of the activity after heating at 60°C for 10 min is observed, thus supporting the above view.

This view was further confirmed by the fact that the thermal stability afforded to carboxypeptidase A by L-phenylalanine in the presence of hippuric acid depends upon their molar ratios (table 1). At the hippuric acid to L-phenylalanine ratio of 1:1 the activity left of $CPAb_1$ and $CPAb_2$ is 55% and 54% respectively as against 10% and 12% respectively at 4:1. This may be compared with 80% retention in the presence of an optimal concentration of L-phenylalanine and absence of hippuric acid.

One can easily rationalise the slow release of phenylalanine as it can bind to the active site of the enzyme by electrostatic forces as shown in figure 5. The low level of protection provided with the substrate hippuryl-L-phenylalanine to $CPAb_1$ and $CPAb_2$ suggests that the actual protection against inactivation is probably provided by L-phenylalanine which is released during substrate hydrolysis and is the last product released by the enzyme so that in steady state, a part of the enzyme remains complexed with L-phenylalanine and this complex remains stable when heated at 60°C.

Enz + hippuryl-L-phenylalanine ⟶ E-hippuryl-L-phenylalanine ─┐
E + L-phenylalanine ⟵ E-L-phenylalanine + Hippuric acid ⟵┘

Such a mechanism differs from the mechanism suggested by other workers (Lipscomb, 1970; Kaiser and Kaiser, 1972; Mock, 1975) in which no acyl intermediate is visualised. All attempts, so far, to isolate such an acyl intermediate have failed (Tomalin *et al.*, 1970). The enzyme shows neither transpeptidase activity (Ginodman *et al.*, 1966) nor gets inactivated by the presence of methanol in the reaction mixture (Hartsuck and Lipscomb, 1971). This proposed mechanism can explain these anomalies. Nuclear magnetic resonance, circular dichroism and kinetic investigations in support of this mechanism are in progress in this laboratory.



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Mechanistic studies on carboxypeptidase A from goat pancreas—Part II: Evidence for carboxyl group

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Abstract. Studies with substrate analogues and the pH optimum indicated the involvement of carboxyl group in the active site of goat carboxypeptidase A. Chemical modification of the enzyme with 1-cyclohexyl-3-(2-morpholinoethyl) carbodiimide metho-*p*-toluene sulphonate, a carboxyl specific reagent, led to loss of both esterase and peptidase activities. Protection studies showed that this carboxyl group was in the active site and was protected by β-phenylpropionic acid and glycyl-L-tyrosine. Kinetic studies also confirmed the involvement of carboxylic group because the enzyme modification with water soluble carbodiimide was a two step reaction which excluded the possibility of tyrosine or lysine which are known to give a one step reaction with this reagent.

Keywords. Active site; carboxyl group; carboxypeptidase A; chemical modification; goat pancreas.

Introduction

Lipscomb *et al.* (1968) from X-ray studies and Bradshaw *et al.* (1969) from primary structure determination of bovine carboxypeptidase A suggested the presence of two carboxyl groups in the active site of the enzyme namely Glu-72 and Glu-270. Petra (1971) from chemical modification with Woodward's reagent *K* concluded that one carboxyl group is involved in the enzyme catalysis. However, Woodward's reagent *K* is very unstable in aqueous medium with half life of only 2·8 min (Dunn and Anfinsen, 1974) which makes the interpretation of the data difficult. Carboxypeptidase A has been purified in this laboratory and further resolved into $CPAg_1$ and $CPAg_2$ isozymes (Dua and Ralhan, 1981a). The goat enzyme is free from kinetic anomalies observed in the case of bovine enzyme (Vallee *et al.*, 1968; Davies *et al.*, 1968; Auld and Vallee, 1971). The goat enzyme differs from the bovine one in N-terminal amino acid, and product stabilisation (Dua and Ralhan, 1981b). Species difference have also been observed in the case of carboxypeptidase A from yeast (Kuku *et al.*, 1976), *Bacillus subtilis*, *Bacillus stearothermophilus* (Waxman and Strominger, 1980), *Streptomyces* R_{61} (Georgopapakadu *et al.*, 1981) which unlike bovine enzyme has serine in the active site.

(Dua and Gupta, 1985). For this report one of the goat isozymes CPAg$_1$ has been chosen for investigating the role of carboxyl group. The enzyme has been modified with 1-cyclohexyl-3-(2-morpholinoethyl) carbodiimide metho-*p*-toluene sulphonate (CMC), a carboxyl group specific reagent (Horae and Koshland, 1967). This reagent is comparatively more stable in aqueous medium which makes interpretation of the kinetic data easier.

Materials and methods

NCbz-glycyl-L-phenylalanine, β-phenylpropionic acid, cyclohexyl-3-(2-morpholinoethyl) carbodiimide (CMC), 2-(N-morpholino) ethane sulphonic acid (MES), and hippuryl-L-lactate were purchased from Sigma Chemical Company, St. Louis, Missouri, USA. N,N′-methylene bisacrylamide, ninhydrin, and methyl cellosolve were procured from M/s. E. Merck, Germany. Bovine serum albumin was from M/s. Fluka, Switzerland. All other reagents were of analytical reagent grade. The solutions were made in deionised water. Ninhydrin was recrystallised from alcohol before use.

Goat carboxypeptidase Ag$_1$ was purified as reported earlier (Dua and Ralhan, 1981a). Carboxypeptidase A activity was determined colorimetrically using NCbz-glycyl-L-phenylalanine as a substrate (Folk and Glander, 1958) while esterase activity was assayed spectrophotometrically using hippuryl-L-phenylactic acid by the method of McClure *et al.* (1968). Protein was determined colorimetrically by the method of Lowry *et al.* (1951) and spectrophotometrically by the method of Warburg and Christian (1941). Spectrophotometric studies were made using a Pye-Unicam SP 500 spectrophotometer.

Ninhydrin solution

Ninhydrin (8·9 g) was dissolved in 250 ml of methyl cellosolve which was freshly distilled after refluxing over solid ferrous sulphate for 3 h. In a separate beaker, citric acid (10·5 g) was dissolved in 100 ml of 1 N NaOH solution. The two solutions were mixed and the pH of the solution was adjusted to 5·6 and its volume was made to 500 ml with water.

Modification of CPAg$_1$ with CMC

Aliquots (4·0 ml, protein: 0·5 mg/ml) were incubated with 20 mM CMC (unless stated otherwise) in 50 mM MES-500 mM sodium chloride buffer, pH 6·0 (unless indicated otherwise), at 4°C for 60 min, desalted on Sephadex G-25 column (0·9 × 60 cm) and assayed for peptidase and/or esterase activity.

Results and discussion

The goat carboxypeptidase Ag$_1$ hydrolyzes acyl dipeptides at fairly rapid rates while the corresponding dipeptides are hydrolysed only slowly (table 1). This may be due

Table 1. Effect of free N-terminal amino group on the rates of hydrolysis of peptides by goat carboxypeptidase Ag_1.

Substrates	Peptidase activity (units/mg)
NCbz-Gly-L-Phe	87·5
Gly-L-Phe	5·7

The assay mixture consists of 1·2 ml of 25 mM of the respective substrates made in 20 mM Tris-100 mM sodium chloride buffer, pH 7·5, and 0·2 ml of enzyme solution (protein: 0·13 mg/ml). The mixture was incubated at 30°C for 10 min and the released amino acid was determined with ninhydrin at 565 nm.

to the formation of an electrostatic bond between free amino group of the substrate and an anionic group such as carboxyl group of the active site thus making the carboxyl group unavailable for catalysis. Such an eventuality will give reduced rates of reaction. This view was confirmed when treatment of $CPAg_1$ solution with CMC led to enzyme inactivation. The enzyme inactivation with CMC was pH (figure 1) and reagent concentration dependent (figure 2). The loss in activity on reacting the enzyme with CMC at pH 6·0 was 78 % in 1 h as compared to no loss at pH 8·0 in the same period. pH

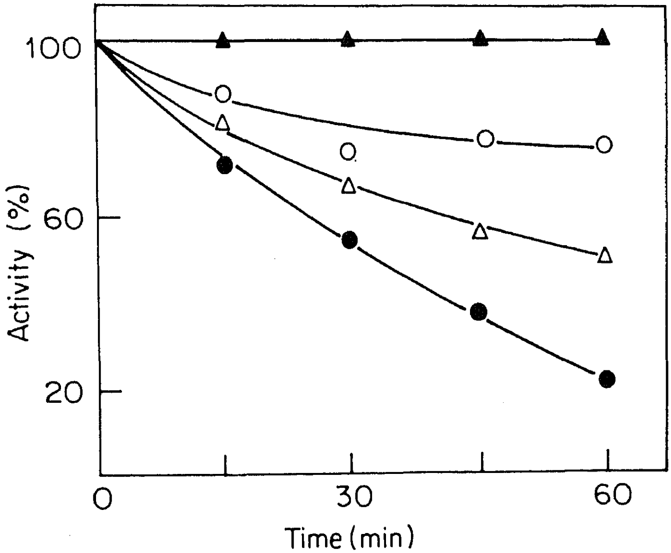

Figure 1. Effect of pH on the modification of goat carboxypeptidase Ag_1 by CMC. Aliquots of the enzyme solution (1 ml, protein: 0·69 mg/ml) in MES buffer were incubated at different pH values in the presence of 20 mM CMC at 4°C for 1 h and peptidase activity was determined

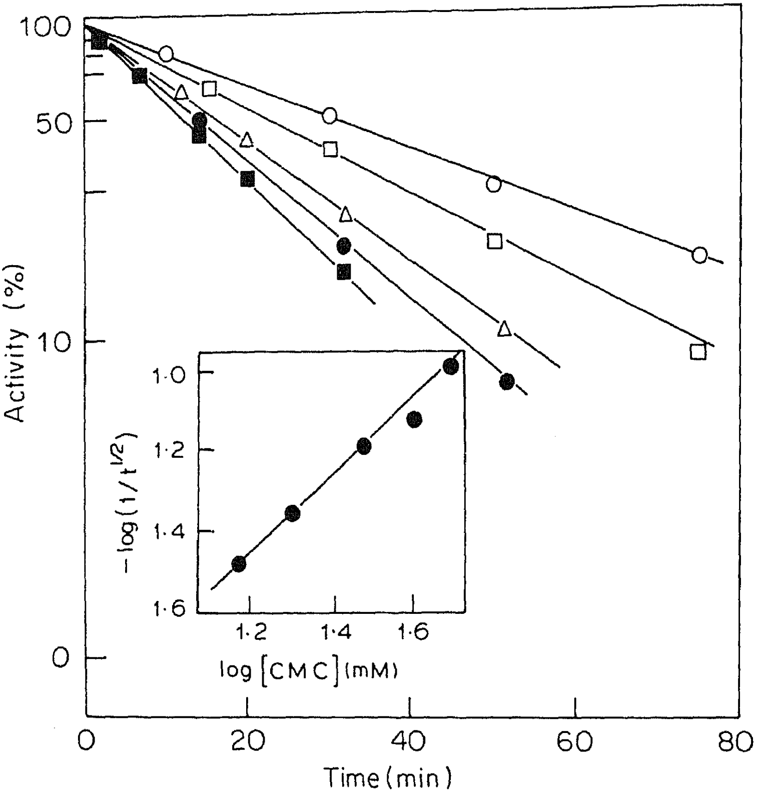

Figure 2. Effect of concentration of CMC on modification of goat carboxypeptidase Ag_1. The enzyme solution (8 ml, protein: 0·5 mg/ml) was modified with 15 mM (○); 20 mM (□); 30 mM (Δ); 40 mM (●) and 50 mM (■) CMC in 50 mM MES-500 mM sodium chloride buffer, pH 6·0, at 4°C. 1·5 ml aliquots were withdrawn at regular time intervals, desalted on Sephadex G-25 column (0·9 × 60 cm) and assayed for peptidase activity. Control was also incubated under similar conditions without CMC and per cent activity left in each sample was calculated by taking control as 100. *Inset*: Apparent order of reaction.

values lower than 6·0 could not be used, as controls kept at pH values below 6·0 showed considerable loss in activity due to denaturation.

The optimal loss in activity on reacting with CMC at pH 6·0, indicates that active site carboxyl group of $CPAg_1$ is being modified and excludes other likely reacting amino acid residues such as lysine, serine/threonine and tyrosine, as their side groups do not act as nucleophiles at this pH. The rate of enzyme modification increased with reagent concentration and showed pseudo-first order kinetics at all the reagent concentrations used. The modified enzyme on exhaustive dialysis showed no recovery of the peptidase as well as esterase activities thus indicating that the loss of activity is

Effect of buffer on the modification of $CPAg_1$ by CMC

Solutions of the enzyme and CMC were made either in 50 mM phosphate, 50 mM acetate, or 50 mM MES buffer. In all the cases, the pH was adjusted to 6·0. The reaction mixture was kept at 4°C for 60 min, desalted on Sephadex G-25 column and assayed for peptidase activity. Maximum inactivation was obtained in 50 mM MES buffer, pH 6·0. Higher concentration of acetate ions and phosphate ions effectively slowed down the reaction and hence, these buffers were avoided for further studies.

Kinetics of modification of $CPAg_1$ with CMC

The half time of inactivation of $CPAg_1$ was calculated from figure 2 and a plot of $-\log(1/t_{0\cdot5})$ *vs* log CMC concentration was drawn (Levy *et al.*, 1963). A slope of 0·86 was obtained (inset figure 2) which suggested that one molecule of the modifier reacted with one molecule of the enzyme protein.

When observed pseudo-first order rate constants (k_{obs}) were plotted against CMC concentration (figure 3) a non-linear plot was obtained, suggesting a two step reaction (Strickland *et al.*, 1975). This also eliminates the possibility of a reaction between tyrosine, lysine or serine residues of the protein as in model experiments these amino acids react with CMC in a one step reaction (Khorana, 1953).

Esterase activity

$CPAg_1$ has both esterase and peptidase activities and hence the question arises whether the active site carboxyl group plays a role in the hydrolysis of both or one type of

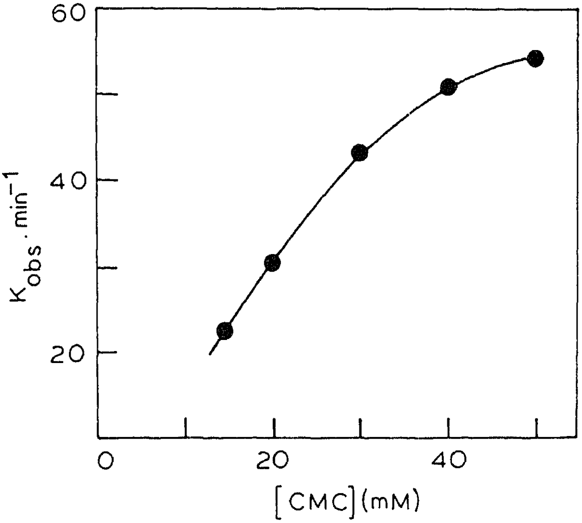

Figure 3. Direct plot of observed pseudo-first order rate constants *vs* concentration of CMC. Observed pseudo-first order rate constants (k_{obs}) were calculated for 15 mM, 20 mM,

that carboxyl group is involved in the hydrolysis of both ester and peptide substrates.

Protection against CPAg$_1$ modification by CMC

The enzyme was modified in the presence and absence of β-phenylpropionic acid, a specific competitive inhibitor of carboxypeptidase Ag$_1$ and glycyl-L-tyrosine, a poor substrate. The enzyme modified in the absence of the inhibitor retains only 19·5% peptidase and 21% esterase activities against 99% in its presence in both cases indicating that β-phenylpropionic acid protects the enzyme against modification (table 2). Similar results were also obtained with glycyl-L-tyrosine. β-Phenylpropionic acid can act as nucleophile and interfere with CMC modification. The enzyme was, therefore, modified in the presence of the same level of sodium acetate concentration. It failed to provide any protection against CMC modification of the enzyme. Thus the protection provided by β-phenylpropionic acid is due to binding at the active site and not due to its nucleophilic attack on CMC and hence it was concluded that CPAg$_1$ has a carboxyl group at or near the active site and modification of a single carboxyl group leads to complete loss of both peptidase and esterase activities.

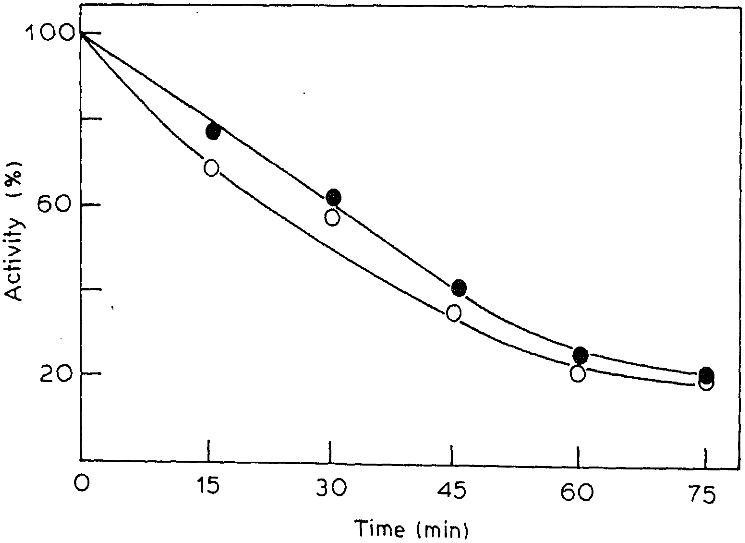

Figure 4. Effect of CMC on esterase activity of goat carboxypeptidase Ag$_1$. The enzyme solution (4 ml, protein: 0·69 mg/ml) was modified with 20 mM CMC in 50 mM MES-500 mM sodium chloride buffer, pH 6·0, at 4°C. 0·7 ml samples were withdrawn at regular time intervals, desalted on Sephadex G-25 column (0·9 x 60 cm) and assayed for peptidase as well as esterase activities. The control was also run under the same conditions in the absence of CMC and per cent activity left in each sample was calculated by taking the control as 100. (O), Peptidase activity; (●), esterase activity.

Table 2. Effect of competitive inhibitors on the modification of goat carboxypeptidase Ag_1 by CMC.

Treatment	Peptidase activity (%)	Esterase activity (%)
Untreated	100·0	100·0
Modification with 20 mM CMC	19·5	21·0
Modification with 20 mM CMC in the presence of β-phenylpropionic acid	98·3	99·1
Modification with 20 mM CMC in the presence of glycyl-L-tyrosine	92·0	95·0

2·0 ml of enzyme aliquots (protein: 0·69 mg/ml), preincubated with 20 mM β-phenylpropionic acid or 20 mM glycyl-L-tyrosine at 30°C for 30 min, were treated with 20 mM CMC in 50 mM MES-500 mM sodium chloride buffer, pH 6·0, at 4°C for 60 min, and desalted on Sephadex G-25 column (0·9 × 60 cm), dialysed and assayed for peptidase activity. Per cent activity left was calculated by taking the activity of the control as 100.

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Effect of cupric-isonicotinic acid hydrazide complex on avian myeloblastosis virus reverse transcriptase and its associated enzyme activities

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Abstract. Cupric complex of isonicotinic acid hydrazide inhibits DNA synthesis by avian myloblastosis virus reverse transcriptase. This inhibition occurs in the presence of either ribonucleotide or deoxyribonucleotide templates. The inhibition of reverse transcriptase by cupric-INH complex is considerably reduced when stored or proteolytically cleaved enzyme was used in the reaction. The complex also inhibits the reverse transciptase-associated RNase H activity. The cupric-isonicotinic acid hydrazide complex cleaves pBR 322 from I DNA into smaller molecules in the presence or absence of reverse transcriptase-associated endonuclease. However, in the presence of the enzyme the DNA is cleaved to a greater extent.

Keywords. AMV reverse transcriptase; cupric complex of isonicotinic acid hydrazide; proteolytic cleavage; RNase H; DNA endonuclease.

Introduction

Reverse transcriptase, a zinc metallozyme, is the key enzyme in the life cycle of retroviruses (Poiesz *et al.*, 1974). The biochemical and immunological properties of this enzyme have been extensively reviewed (Temin and Baltimore, 1972; Gerard and Grandgenett, 1980; Verma, 1981). The major molecular form of avian retrovirus reverse transcriptase is a heterodimer consisting of two structurally related polypeptides α (M_r 63,000) and β (M_r 94,000). The reverse transcriptase of avian type C retroviruses has been shown to possess RNA-dependent DNA polymerase, DNA-dependent DNA polymerase, RNase H and DNA endonuclease activities. Manipulation of any one of these enzyme activities would lead to perturbation in the virus replication cycle. Several chelating agents such as 1, 10-phenanthroline (Modak and Srivastava, 1979), N-methyl isatin β-thiosemicarbazone (Kaska *et al.*, 1978), phosphonoacetic acid (Allaudeen and Bertino, 1978), phosphonoformic acid (Sundquist and Oberg, 1979) and isonicotinic

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Abbreviations used: INH, Isonicotinic acid hydrazide; AMV, avian myeloblastosis virus, SDS-PAGE, sodium dodecyl sulphate polyacrylamide gel electrophoresis; RNase H, ribonuclease H; PMSF, phenyl-transcriptase by cupric-isonicotinic acid hydrazide (INH) complex (Vasudevachari and Antony, 1982). This inhibition was shown to be the result of the preferential binding of the complex to the enzyme. In order to further elucidate the mechanism of this inhibition we have studied the effect of cupric-INH complex on $\alpha\beta$ and α forms of AMV reverse transcriptase, reverse transcriptase-associated RNase H and DNA endonuclease.

Materials and methods

Chemicals

Isonicotinic acid hydrazide, deoxyribonucleoside triphosphates, α-chymotrypsin and phenylmethylsulphonyl fluoride were obtained from Sigma Chemical Co., St. Louis, Missouri, USA. Trypsin-TPCK was purchased from Worthington Biochemical Corp. New Jersey, USA. The synthetic template-primers were obtained from P-L Biochemicals, Milwaukee, USA. [methyl-^{3}H] dTTP (47 Ci/mmol) and [8-^{3}H] dGTP (10 Ci/mmol) were obtained from Amersham International Ltd. Amersham, UK, and fd DNA: [^{3}H] RNA hybrid was a gift from Dr. M. J. Modak, Sloan Kettering Institute for Cancer Research, New York, USA. All other chemicals used were of analytical grade.

Source of chicks and virus has been previously described (Vasudevachari and Antony, 1982). Preparation of cupric-INH complex and the purification of reverse transcriptase were as described earlier (Vasudevachari and Antony, 1982).

Reverse transcriptase assay

The assay was carried out in a final volume of 100 μl and contained the following components: 50 mM Tris-HCl (pH 7·8), 1 mM dithiothreitol, 50 μg of bovine serum albumin, 20 μM [^{3}H]-dTTP (final specific activity of 1000 c.p.m/pmol), 0·5 μg of template-primer, 100 mM KCl, 10 mM $MgCl_2$ and 2 units of enzyme (one unit of enzyme is defined as that amount which incorporates one nanomole of dTMP into an acid-insoluble product in 10 min at 37°C). Incubation was done at 37°C for 30 min. The reaction was stopped by the addition of 5 % (w/v) trichloroacetic acid (TCA) containing 0·01 M sodium pyrophosphate. The acid-insoluble precipitate was collected on Whatman GF/C filters, washed extensively with TCA-containing pyrophosphate, water and finally with ethanol, dried and counted in toluene-based scintillation fluid (Modak and Srivastava, 1979). The same amount of enzyme (0·2 μg/100 μl assay) was used in all the experiments with freshly made, stored or proteolytically cleaved enzyme.

pBR 322 DNA

Plasmid DNA was prepared from *Escherichia coli* HB 101 harbouring pBR 322 by the modified method of Birnboim and Doly (1979) as described by Thomas *et al.* (1983).

Agarose gel electrophoresis

Horizontal agarose gel electrophoresis was carried out using Tris-acetate-EDTA buffer (pH 8·3) as described by Hayward and Smith (1972). After electrophoresis the gels were soaked in the same buffer containing ethidium bromide (0·5 μg/ml) for 30 min before it was examined using an ultraviolet transilluminator.

Polyacrylamide gel electrophoresis

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) was carried out on 10% polyacrylamide gels by the method of Laemmli (1970). The proteins in the samples were precipitated with TCA (10%) before subjecting to electrophoresis. The gels were stained with silver nitrate as described by Merril *et al.* (1981).

Proteolytic cleavage of reverse transcriptase

The proteolytic cleavage of AMV reverse transcriptase was carried out by the method described by Grandgenett *et al.* (1980). Freshly purified AMV reverse transcriptase (16 μg) was digested with 2 μg of trypsin-TPCK or α-chymotrypsin at 37°C in a reaction mixture of 40 μl containing 50 mM Tris-HCl, pH 7·5, 50 mM KCl, 0·02% NP-40, 5 mM DTT, 0·1 M EDTA and 10% glycerol. An aliquot of 5 μl was removed from the reaction mixture at various time intervals and an equal volume of chilled 3 mM PMSF (made in 50 mM Tris-HCl, pH 7·5 containing 2 mM DTT, 0·02% NP-40, 0·1 mM EDTA and 10% glycerol) was added to terminate the reaction. The samples were subjected to SDS-PAGE on a 10% polyacrylamide gel to monitor the cleavage as a function of time. The enzyme cleaved for 5 min with chymotrypsin or 10 min with trypsin was used in further experiments.

RNase H assay

The RNase H assay was carried out according to the method described by Modak and Srivastava (1979). The reaction mixture in a final volume of 100 μl contained 50 mM Tris-HCl (pH 7·8), 1 mM DTT, 50 μg BSA, 10 pmol of fd DNA: [^{3}H] RNA hybrid ($\sim$12,000 cpm) as substrate, 150 mM KCl and 5 mM $MgCl_2$. Reaction was initiated by the addition of 5 units of AMV reverse transcriptase and incubated at 37°C for 30 min. The reaction was terminated by the addition of 5% (w/v) TCA containing 0·01 M sodium pyrophosphate.

Isolation of AMV 70S RNA

AMV 70S RNA was isolated by the method described by Stephenson *et al.* (1972). The RNA was separated on 5–30% linear sucrose gradient by centrifugation at 100,000 *g* for 5 h using a Beckman SW 50·1 rotor.

DNA endonuclease assay

Effect of template-primers on the inhibition of reverse transcriptase by cupric-INH complex

Different template-primers (0·5 μg/100 μl assay) were used in the reaction mixtures with and without cupric-INH complex. The cupric-INH complex inhibited the reverse transcriptase with all the template-primers used (table 1). The inhibition was marginally higher with ribonucleotide templates when compared to the inhibition with deoxyribonucleotide templates.

Table 1. Effect of various template-primers on the inhibition of AMV reverse transcriptase by cupric-INH complex.

		Cupric-INH complex (μM)		
Template-primer	Substrate	0	25	50
Poly(rA)·(dT)$_{12-18}$	[^{3}H]-dTTP	9854	1380 (86)	493 (95)
Poly(rC)·(dG)$_{12-18}$	[^{3}H]-dGTP	16831	3198 (81)	1683 (90)
Poly(rC^m)·(dG)$_{12-18}$	[^{3}H]-dGTP	7530	1807 (76)	979 (87)
Poly(dA)·(dT)$_{12-18}$	[^{3}H]-dTTP	2521	1311 (48)	958 (62)
Poly(dC)·(dG)$_{12-18}$	[^{3}H]-dGTP	2901	1305 (55)	899 (69)
AMV-70S RNA	dATP, dCTP, dGTP,[^{3}H]-dTTP	1384	581 (58)	374 (73)

Values are given as cpm and the values in parentheses indicate per cent inhibition of control.

Effect of cupric-INH complex on αβ and α forms of reverse transcriptase

In our studies, it was observed that the inhibition of reverse transcriptase by cupric-INH complex was markedly reduced when the enzyme was used after storage at −20°C for 8–10 months in phosphate buffer (pH 7·3) containing 50% glycerol. An attempt was therefore made to investigate whether the gradual conversion of αβ holoenzyme to α polypeptide during storage was responsible for this observation. The SDS-PAGE profile of the stored enzyme (figure 1A) showed more α polypeptide and less β polypeptide. The αβ holoenzyme (freshly purified) was subjected to proteolytic cleavage and was used in the cupric-INH complex inhibition studies. Both chymotrypsin (figure 1B) and trypsin (figure 1C) cleaved most of the αβ holoenzyme to α polypeptide without significant loss of polymerase activity in 5 and 10 min respectively. Further cleavage resulted in complete loss of enzyme activity.

The inhibition of reverse transcriptase (freshly purified) by cupric-INH complex was compared with that of the stored, chymotrypsin-cleaved and trypsin-cleaved enzymes (table 2). Cupric-INH complex at 50 μM concentration inhibited the stored, chymotrypsin-cleaved and trypsin-cleaved enzymes to 48, 28 and 20% respectively,

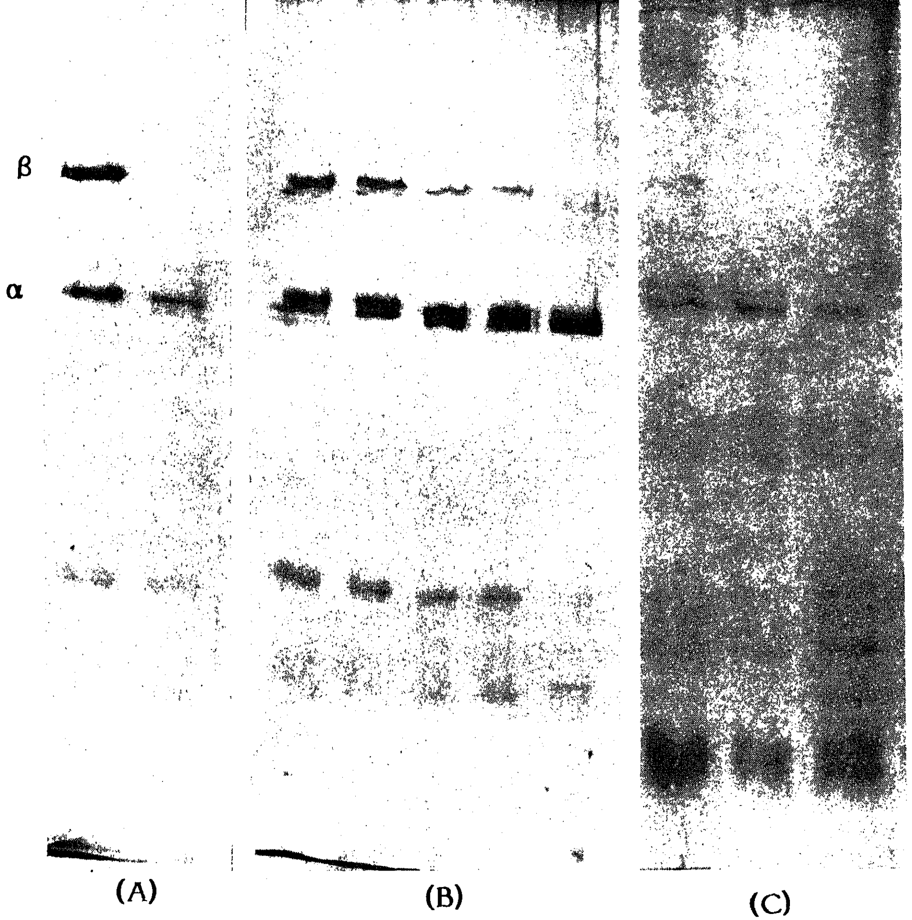


Figure 1. SDS-PAGE pattern. A. Stored reverse transcriptase: lane 1, freshly purified reverse transcriptase; and lane 2, enzyme stored for 8–10 months at −20°C. B. Chymotrypsin-cleaved reverse transcriptase: lanes 1–5, reverse transcriptase incubated with α-chymotrypsin for 0, 1, 2, 3 and 5 min respectively. C. Trypsin-cleaved reverse transcriptase: lanes 1–3, reverse transcriptase incubated with trypsin for 3, 5 and 10 min respectively

Inhibition of RNase H activity by cupric-INH complex

The results of the effect of cupric-INH complex on RNase H activity are shown in table 3. It is clear from the results that the inhibition was dose-dependent. At 20, 50 and 100 μM concentrations of the complex the enzyme activity was inhibited to 62, 74 and 97% respectively.

Table 2. Effect of cupric-INH complex on stored and proteolytically cleaved AMV reverse transcriptase.

Cupric-INH complex (μM)	[^{3}H]dTMP incorporation (cpm)			
	Fresh enzyme	Stored enzyme	Chymotrypsin-cleaved enzyme	Trypsin-cleaved enzyme
0 (Control)	9825	8153	6061	7326
20	2611 (73)	6306 (23)	5008 (17)	5931 (19)
50	450 (95)	4224 (48)	4375 (28)	5862 (20)
100	275 (97)	2863 (65)	3467 (43)	3204 (56)
200	180 (98)	801 (90)	765 (87)	531 (93)

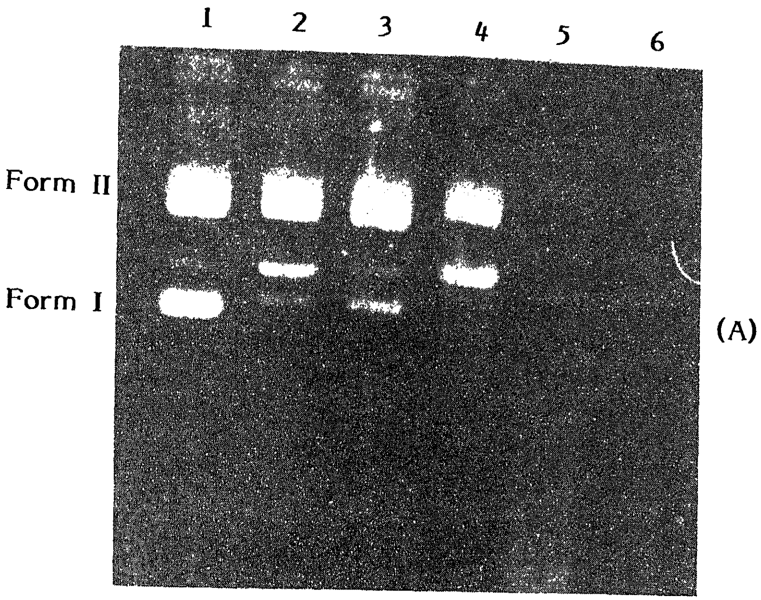
Values in parentheses indicate per cent inhibition of control. In the assay mixture poly(rA).$(dT)_{12-18}$ was used as the template-primer.

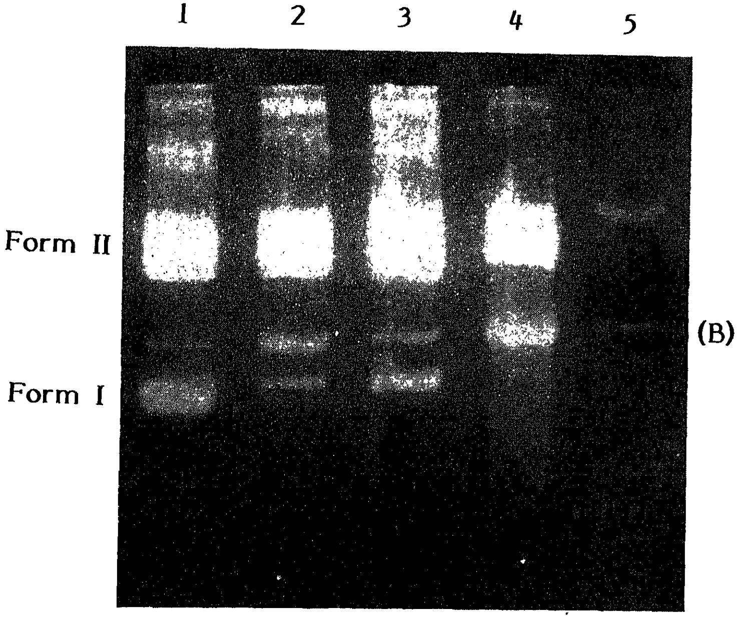
Table 3. Effect of cupric-INH complex on RNase H activity.

Concentration of cupric-INH complex (μM)	[^{3}H]-RNA solubilized (cpm)	Activity (%)
0 (Control)	2757	100
20	1057	38
50	713	26
100	96	3

Effect of cupric-INH complex on DNA endonuclease activity

The effect of cupric-INH complex on DNA endonuclease was studied by using the chymotrypsin-cleaved reverse transcriptase. The reaction was carried out at 37°C using pBR 322 DNA as the substrate and the products were analysed on 1% agarose gels. Preparations of pBR 322 DNA generally give two bands corresponding to covalently closed circular (supercoiled or form I) and relaxed circular (form II) DNA when electrophoresed on 1% agarose gels (figure 2A, lane 1). The extent of conversion of pBR 322 form I DNA to form II by DNA endonuclease was monitored qualitatively by observing the relative intensity of the bands over that of control. The electrophoretic pattern indicated that in 2·5 h the pBR 322 form I DNA was converted to form II DNA to a considerable extent in presence of uncleaved (lane 2) or cleaved reverse transcriptase (lane 3). When the reaction was carried out in presence of cupric-INH complex (100 μM and above) streaks of DNA were observed in the gels showing greater extents of DNA cleavage (lanes 4 to 6).


1
2
3
4
5
6
Form II
Form I
(A)


1
2
3
4
5
Form II
Form I
(B)

enzyme and the products analysed on 1 % agarose gels (figure 2B). It was observed that the cupric-INH complex alone could cleave the DNA but to a lower extent when compared to the cleavage obtained in presence of the enzyme. Higher concentrations (0·5 and 1·0 mM) of the complex were required to obtain the same extent of DNA cleavage (lanes 4, 5) as observed in presence of the enzyme.

Discussion

We have reported earlier that cupric-INH complex inhibited AMV reverse transcriptase noncompetitively and the inhibition was shown to be due to the preferential binding of the complex to reverse transcriptase (Vasudevachari and Antony, 1982). In the present investigation the complex was shown to inhibit the reverse transcriptase in the presence of ribo- or deoxyribonucleotide templates. The inhibition of reverse transcriptase by cupric-INH complex was considerably reduced when stored or proteolytically cleaved enzyme was used in the reaction. The complex was shown to bind to the same extent to both $\alpha\beta$ holoenzyme and chymotrypsin-cleaved enzyme (data not shown). It is observed that the proteolytic cleavage of $\alpha\beta$ holoenzyme to α polypeptide does not significantly reduce the polymerase activity or affect the binidng efficiency of the complex. It may be possible that the complex could bind to both the 32,000 dalton polypeptide ($p32^{pol}$) and the 64,000 dalton α polypeptide as the chymotrypsin-cleavage products were not separated in these reactions.

The active site for RNase H activity and template-primer binding site have been reported to be identical (Modak and Srivastava, 1979; Modak and Marcus, 1977). since RNase H activity was inhibited by cupric-INH complex, it may be suggested that the complex acts at the template-primer binding site of reverse transcriptase. Recently, a dialdehyde derivative of ATP (Srivastava *et al.*, 1983) was shown to inhibit AMV reverse transcriptase by blocking the essential sulphydryl group present at the template-primer binding site of the enzyme protein. Since β-mercaptoethanol was shown to reverse the inhibition of reverse transcriptase by cupric-INH complex (Srivastava *et al.*, 1978), it seems possible that cupric-INH complex mediated inhibition is similar to that shown in the case of dialdehyde-ATP.

The proteolytic cleavage of AMV reverse transcriptase by chymotrypsin generates a polypeptide of M_r 32,000 ($p32^{pol}$) which leads to activation of DNA endonuclease activity (Grandgenett *et al.*, 1980). The effect of cupric-INH complex on the chymotrypsin-activated DNA endonuclease was studied by monitoring the conversion of form I pBR DNA to form II DNA. When cupric-INH complex was included in the reaction, the DNA was cleaved into small fragments giving a streak in the gel. Such a DNA cleavage may not be entirely due to the enahnced endonuclease activity by cupric-INH complex because under these conditions cupric-INH complex by itself caused a similar cleavage of DNA though to a lesser extent. A possible mechanism for the DNA *al.*, 1980; Marshall *et al.*, 1981) However, the biochemical mechanism of DNA cleavage by cupric-INH complex remains to be elucidated.

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α-Galactosidase from germinating guar (*Cyamopsis tetragonolobus*) seeds

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Abstract. The changes in α-galactosidase activity in guar (*Cyamopsis tetragonolobus*) seeds was followed during seven days of germination. The enzyme activity was maximal on the first day of germination and gradually decreased during subsequent days. On the second day of germination the partially purified enzyme upon ion-exchange chromatography on CM-Sephadex C-50 was resolved into α-galactosidase-A (anionic), α-galactosidase-C_1 (cationic) and α-galactosidase-C_2 (cationic) and their relative proportions were 28, 12 and 60%, respectively. The combined α-galactosidase C_1 and C_2 activities increased in the first two days of germination followed by significant decrease after the 3rd day onwards, whereas α-galactosidase-A remained fairly constant throughout the germination period. α-Galactosidase-A and C_2 had different K_m and V_{max} values with *p*-nitrophenyl α-D-galactopyranoside, raffinose and melibiose as substrates and also differed in their thermal stabilities.

Keywords. Guar; *Cyamopsis tetragonolobus*; Leguminoseae, germination; α-galactosidase.

Introduction

α-Galactosidase (α-D-galactoside galactohydrolase EC 3.2.1.22) catalyses the hydrolysis of α-galactosyl linkages present in oligosaccharides, galactomannans and glycoproteins and is found in many leguminous seeds, microorganisms and some animal tissues (Dey and Pridham, 1972). Galactose linked oligo- and polysaccharides occur in a number of leguminous seeds. Especially, galactomannans occur as a major storage polysaccharide in varying amounts in the endosperm of a wide range of leguminous seeds (Anderson, 1949). Galactose content of galactomannans varies depending on the plant source. Not all α-galactosidases hydrolyse galactomannans and those that do, appear to remove the terminal galactose residue only. The mature seeds of guar contain a huge reserve of galactomannan and to a lesser extent other galacto-oligosaccharides (Anderson, 1949). On germination the endospermic reserve carbohydrate is degraded and utilized (Dey, 1978). The role of α-galactosidase in the mobilization of reserve galactomannan has been established (Reid and Meier, 1973; McCleary and Matheson, 1974; Williams *et al.*, 1978).

The occurrence of multiple forms of α-galactosidases was first reported from coffee

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Abbreviations used: PNPG, Paranitrophenyl α-D-galactopyranoside; PMSF, phenyl methyl sulphonyl fluoride; PVP, polyvinyl pyrrolidine.

beans by chromatography on alumina columns (Petek and Dong, 1961). Since then a number of reports from plant and microbial sources have indicated the existence of multiple forms of α-galactosidase (Dey and Pridham, 1968; Pridham and Dey, 1974; Barham *et al.*, 1971; Williams *et al.*, 1978; Lee and Wacek, 1970; Suzuki *et al.*, 1970).

The present work demonstrates the existence of multiple forms of α-galactosidase from 2-day germinated guar seeds and their relative proportions during different stages of germination and describes some of the kinetic properties.

Materials and methods

Guar (*Cyamopsis tetragonolobus*) seeds were obtained from Haryana Seed Corporation, Hissar. CM-Sephadex C-50, melibiose, raffinose, paranitrophenyl α-D-galactopyranoside (PNPG), phenyl methyl sulphonyl flouride (PMSF), insoluble polyvinyl pyrrolidine (PVP) were products of Sigma Chemical Co., St. Louis, Missouri, USA. All other chemicals and solvents used were of analytical grade. Unless and otherwise mentioned all the isolation and fractionation steps were carried out at 4°C.

Isolation and fractionation of α-galactosidase

Guar seeds were surface sterilized with 0·1 % $HgCl_2$ solution, washed and imbibed with distilled water for 4 h. Germination was carried out in the dark at 25°C for 1–7 days on filter paper moistened with distilled water. At the end of germination period, the seeds were dehusked and chilled. The enzyme was extracted from the chilled seedlings by homogenizing with 5 volumes of 50 mM sodium acetate buffer pH 5·5 containing 2 mM EDTA, 2 mM β-mercaptoethanol, 1 mM PMSF, 200 mM NaCl and 1% insoluble PVP. The slurry was filtered through a mull cloth and the filtrate was centrifuged at 5000 *g* for 20 min. The crude extract which had been brought to 40% $(NH_4)_2SO_4$ saturation was kept for 4 h and centrifuged at 12,000 *g* for 20 min. The supernatant which had all the α-galactosidase activity was adjusted to 70% $(NH_4)_2SO_4$ saturation and kept for 4 h and centrifuged at 12,000 *g* for 20 min. The precipitate was dissolved in a minimum volume of 50 mM acetate buffer pH 5·5 and dialysed against 2 litres of 5 mM acetate buffer (pH 5·5) overnight with two changes. The dialysed enzyme was adjusted to 50% (v/v) acetone saturation and kept for 30 min at 4°C and centrifuged at 12,000 *g* for 20 min. The precipitate was dissolved in a minimum volume of 50 mM acetate buffer (pH 5·5) and dialysed against 50 mM acetate buffer (pH 5·0) overnight with two changes. The enzyme thus obtained had a specific activity of 2·8.

Enzyme assay

The enzyme activity was determined according to the procedure of Suzuki *et al.* (1970) with minor modifications. The enzyme was assayed by incubating 2 μmol of PNPG, 25 μmol of acetate buffer pH 5·5 and 0·1 ml of suitably diluted enzyme in a total volume

Results and discussion

Changes in total α-galactosidase activity during germination

A germination profile was carried out for 7 days to study the changes in the total α-galactosidase activity in the crude extracts of 100 uniformally germinated seedlings on each day under identical conditions. The change in total α-galactosidase activity is shown in figure 1. The levels of total α-galactosidase activity reached a maximum after 24 h of germination and decreased gradually upto 4th day and there was a rapid decrease in the activity on the 5th and 6th day of germination. Since the level of α-galactosidase activity was low in dry seeds, it was clear that α-galactosidase activity increased on germination until the depletion of galactomannan, which is a major storage polysaccharide in guar, and then decreased.

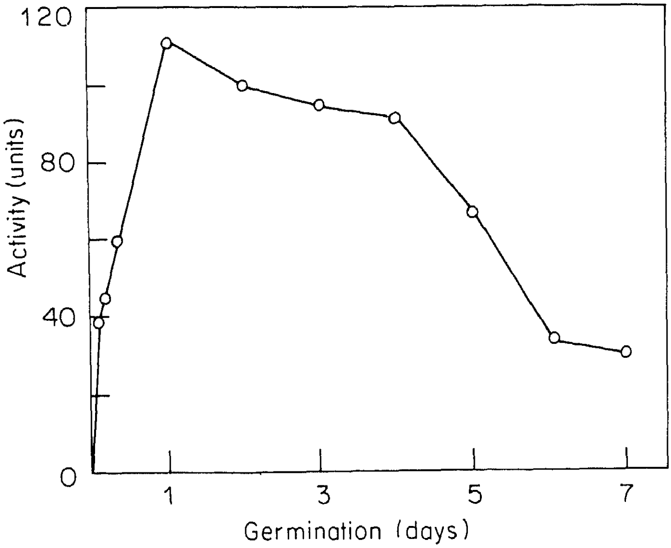

Figure 1. Changes in total α-galactosidase activity during germination.

Multiple forms of α-galactosidases

Acetone precipitated enzyme from 2-day old germinated seedlings was subjected to further fractionation by ion-exchange chromatography on CM-Sephadex C-50 column (3 × 70 cm) which had been equilibrated with 50 mM acetate buffer, pH 5·0. The flow rate was adjusted to 40 ml/h and 8 ml fractions were collected. After loading the enzyme, the column was washed with equilibration buffer and subjected to a step-wise elution with 0·2 M and 1·0 M NaCl in equilibration buffer. As shown in figure 2,

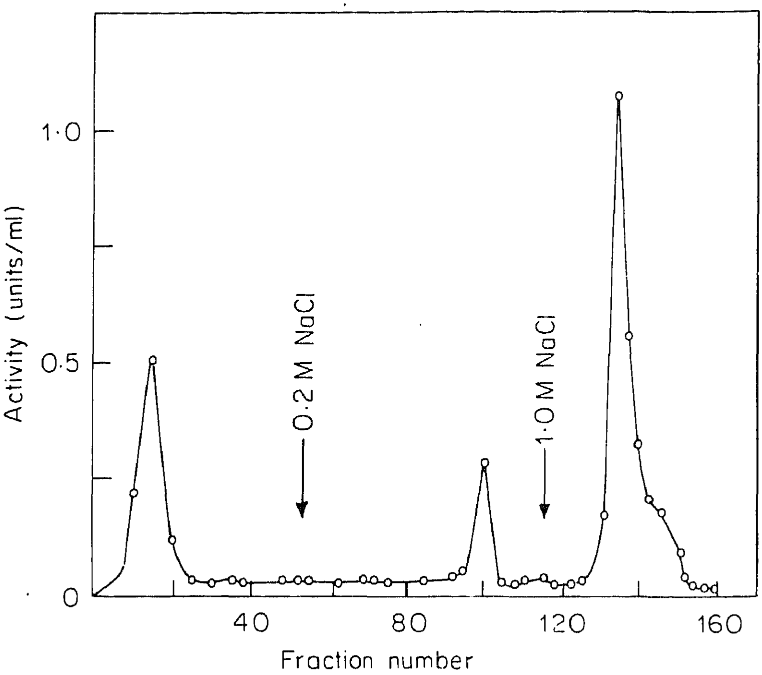

Figure 2. Elution profile of α-galactosidase on CM-Sephadex C-50 column.

chromatography of α-galactosidase on CM-Sephadex C-50 gave three distinct active fractions termed α-galactosidase-A (anionic, 28%) which comes out with buffer washings and strongly binds to DEAE-Sephadex A-50 at pH 7·0, α-galactosidase C_1 (cationic, 12%) eluted at 0·2 M NaCl concentration and α-galactosidase C_2 (cationic, 60%) eluted at 1·0 M NaCl concentration.

McCleary and Matheson (1974) reported the presence of only two forms of α-galactosidases-A and C from extracts of six days old germinated guar seedlings by chromatography on DEAE-Cellulose column. The reason for their failure to detect three forms of α-galactosidases may be due to the fact that the enzyme was isolated after six days of germination when the enzyme was at its minimum level. Further in the same study they have also indicated that a different species of guar (*Cyamopsis psoraloides*) contained three forms of α-galactosidase, although no detailed information was given. Eventhough there are a good number of reports on the existence of multimolecular forms of α-galactosidase in a variety of leguminous seeds with different physico-chemical properties, their specific role during germination is not clearly understood. However, some evidence for the inter-conversion of multimolecular forms of α-galactosidase *in vitro* has been demonstrated in the case of *Viciafaba* enzyme by Dey *et*

Table 1. Kinetic properties of multimolecular forms of α-galactosidase from germinated guar seeds.

	pH optimum	Temperature optimum (°C)	K_m (mM) and $V_{\max}$ (μmol/min/mg)					
			PNPG		Melibiose		raffinose	
			k_m	$V_{\max}$	K_m	$V_{\max}$	K_m	$V_{\max}$
α-Galactosidase-A	5·5	60	1·20	5·75	4·6	0·08	10·0	0·32
α-Galactosidase C_1	5·5	70	0·25	9·25	11·1	1·42	10·0	3·75
α-Galactosidase C_2	5·5	70	0·50	4·76	5·6	1·31	14·3	25·35

determined using PNPG as substrate under standard assay conditions. All the three forms of α-galactosidase had a sharp pH optima of 5·5, similar to the enzymes from coffee beans (Petek and Dong, 1961), *S. oleracea* (Gatt and Baker, 1970), *Stachs offinis* (Ueno *et al.*, 1980) and *Saccharum afficinarum* (Chinen *et al.*, 1981). It was observed that α-galactosidases from guar exhibited a very high temperature optimum. α-galactosidase-A had an optimum temperature of 60°C whereas α-galactosidase C_1 and C_2 had an optimum temperature of 70°C similar to the enzyme from *Saccharum officinarum.*

The effect of substrate concentration on the enzyme activity was studied using *p*-nitrophenyl α-D-galactopyranoside (0·1–10 mM), melibiose and raffinose (0·5–40 mM). The reaction was carried out for 5 min at 60°C in 50 mM acetate buffer pH 5·5, in the case of PNPG the rate of hydrolysis was determined as described under materials and methods. In the case of melibiose and raffinose the reaction was carried out for 30 min at 60°C (pH 5·5) and followed by determining the liberated glucose by modified glucostat reagent method (Dahlqvist, 1951) and the liberated galactose by the method of Nelson (1944). There was no inhibition by any of these substrates at the above mentioned concentrations. The K_m and $V_{\max}$ values calculated from Lineweaver-Burk plot are given in table 1.

Thermal stability

α-Galactosidases exhibit varying degree of stability to heat depending on their origin (Dey and Pridham, 1972). Thermal stabilities were carried out in 50 mM acetate buffer pH 5·5 at temperatures ranging from 40–70°C over a period of 30 min and the residual activity was determined using PNPG as substrate. The pattern of thermal stability of α-galactosidase-A, C_1 and C_2 are shown in figure 3. Guar α-galactosidases exhibited fairly high degree of thermal stability. All the three forms of enzymes were heat stable upto 45°C. α-Galactosidases C_1 and C_2 were more heat stable compared to α-galactosidase-A. α-Galactosidase-A lost about 28, 58 and 94% of its activity at 50°, 60° and 70°C respectively in 30 min.

Changes in the relative proportions of α-galactosidase-A, C_1 and C_2 during germination

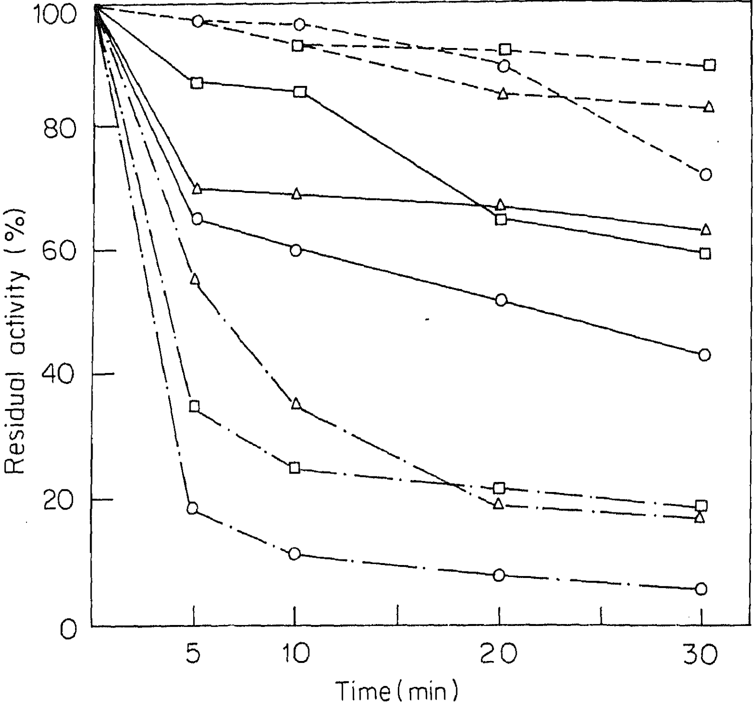

Figure 3. Thermal stability of α-galactosidases. (o), α-Galactosidase-A, (50°C); (□), α-Galactosidase C_1 (60°C); (Δ) α-Galactosidase C_2 (70°C).

was washed with equilibration buffer to remove α-galactosidase-A and then eluted with 1·0 M NaCl in the same buffer to fractionate α-galactosidase C_1 and C_2. The activity profiles of α-galactosidase-A, C_1 and C_2 during germination are shown in figure 4. The combined activities of C_1 and C_2 decreased significantly during germination period whereas the activity of α-galactosidase-A remained fairly constant throughout the germination period.

It can be seen from figure 4 that the total recovery of enzymes from the CM-Sephadex C-50 column on 3rd and 4th day of germination is rather low compared to the level of total enzyme present in the extract (figure 1). The reason for this low recovery is not clear at the moment, however, it is possible that 3rd and 4th day extract contains some labile α-galactosidase C_1 and C_2 and may represent the onset of rapid change in activity after the 3rd day. The fact that the level of α-galactosidase-A is more or less similar on all the seven days of germination clearly suggests that only the α-galactosidase C_1 and C_2 change rather dramatically. Interestingly it has been observed that the galactomannan reserve in guar seed is rapidly reduced between 1st and 2nd day of germination (McCleary, 1983). The α-galactosidase activity is maximal during this period of germination (figure 1). Taken together these results suggest that α-

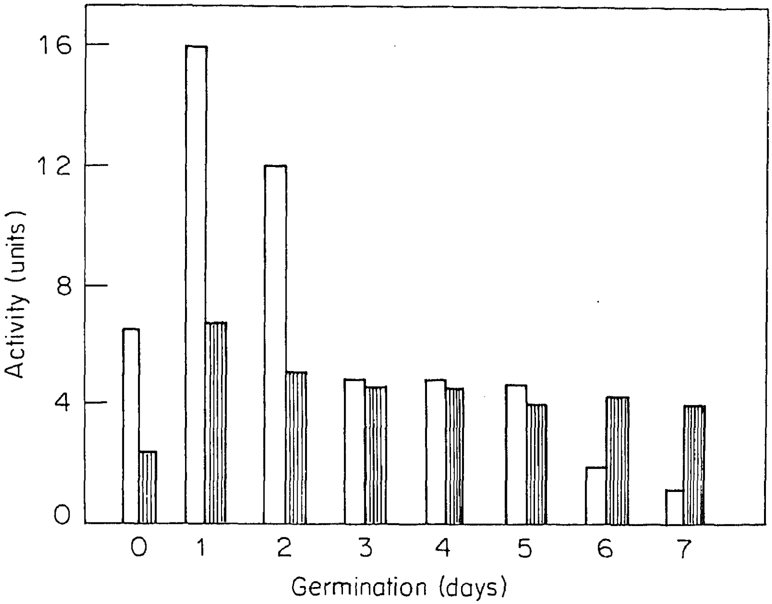

Figure 4. Changes in the α-Galactosidase-A, C_1 and C_2 during germination. (□), α-Galactosidase C_1 and C_2; ▥ , α-Galactosidase-A.

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Lipoic acid and diabetes II: Mode of action of lipoic acid

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Abstract. Intraperitoneal administration of lipoic acid (10 mg/100 g) does not effect changes in serum insulin levels in normal and alloxan diabetic rats, while normalising increased serum pyruvate, and impaired liver pyruvic dehydrogenase characteristic of the diabetic state. Dihydrolipoic acid has been shown to participate in activation of fatty acids with equal facility as coenzyme A. Fatty acyl dihydrolipoic acid however is sparsely thiolyzed to yield acetyl dihydrolipoic acid. Also acetyl dihydrolipoic acid does not activate pyruvate carboxylase unlike acetyl coenzyme A. The reduced thiolysis of β-keto fatty acyl dihydrolipoic acid esters and the lack of activation of pyruvic carboxylase by acetyl dihydrolipoic acid could account for the antiketotic and antigluconeogenic effects of lipoic acid.

Keywords. Lipoic acid; diabetes; acetyl coenzyme A; acetyl dihydro lipoic acid; fatty acid oxidation; gluconeogenesis.

Introduction

The role of lipoic acid as a cofactor in the multienzyme complexes that catalyze the oxidative decarboxylation of α-keto acids such as pyruvate, α-ketoglutarate and branched chain α-keto acids has been established for some time (Reed, 1974). Since lipoic acid can be biosynthesised in the animal (Spoto *et al.*, 1982; Carreau *et al.*, 1977; Carreau, 1979), little attention has been given to a dietary requirement for lipoic acid *per se* or lipoic acid content of dietary constituents, which in any case is relatively low (Herbert and Guest, 1975). In an earlier communication (Natraj *et al.*, 1984) we have shown that lipoic acid levels are reduced in alloxan diabetic rats and administration of lipoic or dihydrolipoic (DTO) acid reduces blood sugar and acetoacetate levels at the same time bringing about an increase in liver glycogen and fat synthesis. The mode of action of lipoic acid was, however, not considered.

It is well known that administration of insulin or oral hypoglycemic agents which stimulate insulin secretion correct the metabolic disorders in diabetes. Therefore, the first thing to look for is whether lipoic acid stimulates insulin secretion and whether the antidiabetic effect of lipoic acid is exerted through insulin. In this study we have shown that lipoic acid administration does not stimulate insulin secretion in normal and diabetic rats ruling out insulin as the mediator of the hypoglycemic effects of lipoic acid.

acid substitution in the enzyme complexes, where it is known to be a cofactor, is reduced. One such enzyme is pyruvate dehydrogenase (PD) (EC 1.2.4.1) which is a crucial enzyme located at the metabolic branch point between carbohydrate, fat and protein metabolisms. In this study we have shown that PD activity is stimulated upon lipoic acid administration and this is reflected in a reduction in serum pyruvate levels.

In diabetes, major impairments in carbohydrate, fat and protein metabolisms occur. Impaired transport of glucose into tissues and decreased activity of hexokinase (May and Mikulecky, 1983) result in reduced utilization of glucose. Much of the energy requirements in the liver and kidney are met by fatty acid oxidation, resulting in increased ketogenesis in these organs (Randle, 1966). Increased fatty acid oxidation also triggers increased gluconeogenesis mainly from lactate, alanine and glutamine leading to protein catabolism and elevated blood sugar levels (Ruderman *et al.*, 1976; Utter *et al.*, 1964). In this paper, we have shown that DTO brings about a reduction in acetoacetate levels in the livers of starved rats perfused with octanoate. We have therefore examined the effect of lipoic acid on some of the key steps of fatty acid oxidation such as fatty acyl coenzyme A (CoA) synthetase (EC 6.2.1.3) and thiolase (EC 2.3.1.9) and found that while fatty acyl DTO is formed readily from fatty acids and DTO, the oxidation of fatty acids is slower in view of the fact that acetoacetyl DTO and perhaps β-ketoacyl DTO is a poor substrate for thiolase.

Increased activity of pyruvate carboxylase (EC 6.4.1.1) has been implicated in the elevated rates of gluconeogenesis in diabetes (Hers and Hue, 1983; Weinberg and Utter, 1980). We present evidence that acetyl DTO differs from acetyl CoA in that it does not stimulate pyruvate carboxylase (PC) thus accounting for the hypoglycemic effect of lipoic acid.

Materials and methods

DL α-lipoic acid and alloxan were purchased from British Drug House, England. Radio immunoassay kit for insulin assay was procured from Bhabha Atomic Research Centre, Bombay. Acetoacetyl CoA, ATP, CoA, bovine serum albumin, diketene, DTNB, DTT, lactate dehydrogenase (LDH) (EC 1.1.1.27), NAD, NADPH and sodium pyruvate were purchased from Sigma Chemical Co., St. Louis, Missouri, USA. All other reagents were of analytical grade. The rats used were an inbred Haffkine Wistar strain maintained on Hindustan Lever pellet feed. Diabetes was induced by a single subcutaneous injection of alloxan (100 mg/kg) dissolved in acetate buffer (0.1 M, pH 5.4). Rats excreting sugar in the urine (2–3 g/dl) were used in this study. Acetoacetyl DTO was prepared according to Drummond and Stern (1961). Acetyl DTO was prepared according to Gunsalus *et al.* (1956).

pyruvic acid levels were estimated according to the method described by Varley *et al.* (1976).

Mitochondrial PD was isolated according to the method of Linn *et al.* (1972). Eighteen normal and 18 diabetic rats were distributed into 3 groups each. One group was injected 0·1 ml saline, a second group sodium lipoate (10 mg/100 g body wt) as a solution in 0·1 ml saline and the third group received insulin (4 I.U./100 g). All injections were intraperitoneal.

Assay of PD was carried out according to the procedure of Stepp *et al.* (1981). The assay mixture contained in a volume of 1 ml, 2·5 μmol of NAD, 0·32 μmol DTT, potassium phosphate buffer (pH 8·0, 50 μmol), 2 μmol pyruvate, 1 μmol magnesium chloride and 0·13 μmol, CoA or DTO. The reaction was started by adding an aliquot of the enzyme (approximately 100 μg protein) and the reduction of NAD monitored at 340 nm. Protein was estimated according to Lowry *et al.* (1951). The product of the reaction with DTO was identified by extracting the reaction mixture with benzene, evaporating the solvent and formation of acetyl hydroxamate according to Lipmann and Tuttle (1945).

Serum insulin levels in normal and diabetic rats

Forty normal and 24 diabetic rats were divided into 2 groups and kept away from food for 3 h. One group was administered intraperitoneally 0·1 ml of saline and the other sodium α-lipoate (10 mg/100 g) dissolved in 0·1 ml saline. After 60 min the rats were sacrificed and the blood was collected in centrifuge tubes. Blood sugar was estimated according to the method of Somogyi (1952). Serum insulin was estimated by the method of Herbert *et al.* (1965) as modified and described in the booklet supplied by Bhabha Atomic Research Centre, Bombay, along with the radioimmunoassay kit for insulin.

Acetoacetate levels in starved rat livers perfused with octanoate

Normal male adult rats weighing about 250 g were starved for 48 h. The rats were anaesthetized with ether and canulated through the portal vein. Livers were perfused for 60 min with oxygenated Krebs bicarbonate buffer containing either 2 mM octanoate or 2 mM octanoate and 2 mM DTO. The perfusion volume was 100 ml. The perfusate was collected and to 5 ml of the perfusate 2 ml of chilled trichloroacetic acid (TCA; 20 %) was added and centrifuged. Acetoacetate in the supernatant was estimated according to the method described by Walker (1954).

Activation of fatty acids with DTO catalyzed by acyl CoA synthetase

The enzyme was isolated from normal rat liver microsomes according to the procedure of Dang *et al.* (1984). The crude microsomal pellet was used as the source of the enzyme.

The assay was performed according to the procedure of Dang *et al.* (1984) with some modifications. The assay mixture in a final volume of 1 ml contained 200 μmol Tris HCl

ATP and incubating for 3 min. Palmitoyl CoA formed was estimated by extracting the acidified reaction mixture with diethyl ether and determining the radioactive counts in the aqueous layer. The palmitoyl DTO formed was estimated by extracting the acidified reaction mixture with ether and the free palmitic acid was separated from the palmitoyl DTO in the ether layer by thin layer chromatography using n-hexane: diethyl ether: acetic acid (80:20:1; v/v) as the solvent system. The band corresponding to palmitoyl DTO was cut out and counted.

The control for the above assay contained all the ingredients except ATP.

Acetoacetyl CoA and acetoacetyl DTO as substrates for thiolase

Thiolase was partially purified from normal rat livers (upto 60% ammonium sulphate fractionation step) according to the procedure of Stern *et al.* (1960). Each assay mixture contained in a final volume of 1 ml: 67 μmol of Tris buffer (pH 8·1) 0·2 μmol acetoacetyl thioester, 1·4 mg enzyme and 0·22 μmol of thiol. The reaction was monitored spectrophotometrically by the decrease in absorbance at 310 nm. All the components with the exception of the acetoacetyl thioester were added to the reference cuvette.

Pyruvate carboxylase and acetyl DTO

PC was isolated from rat liver mitochondria according to the procedure described by Scrutton *et al.* (1969). The assay was carried out by coupling it with citrate synthase according to Martin and Denton (1970).

The assay mixture in a final volume of 1 ml contained: 100 μmol of Tris buffer pH 7·4, 10 μmol of sodium pyruvate, 10 μmol of $MgCl_2$, 1 μmol of ATP, 0·5 μmol of acetyl CoA/acetyl DTO, 30 μmol of sodium bicarbonate, 100 μg of pyruvate carboxylase, 160 μg of citrate synthase and 1 μmol of DTNB. The increase in absorbance at 412 nm was followed spectrophotometrically.

Results

Effect of lipoic acid on blood pyruvate and liver pyruvate dehydrogenase activity in normal and diabetic rats

Serum pyruvate levels are increased in diabetes and after lipoic acid administration, the levels are brought down to near normal values (table 1). This decrease was statistically significant ($P < 0·05$).

Liver PD is 2 times higher in normal rats than in diabetic rats (table 2). In both normal and diabetic rats, lipoic acid administration brought about significant increases in the enzyme activity. The increase was 60% in normal rats and 90% in diabetic rats. This compares with an increase of 74% and 111% observed in normal and diabetic rats respectively, following insulin administration.

The results of substitution of DTO for CoA in the estimation of PD are shown in

Table 1. Effect of lipoic acid on serum pyruvate levels.

Treatment	Mean levels ± SE µmol/litre
Normal	85·4 ± 16·4
Diabetic	137·4 ± 3·7*
Diabetic + Lipoic acid	93·1 ± 17.2**

Lipoic acid was administered (10 mg/100 g) intraperitoneally and serum pyruvate was estimated 60 min later as described in Materials and methods. All the determinations are means of 6 animals.

* Significantly greater than normal $P < 0{\cdot}05$

** Significantly less than diabetic $P < 0{\cdot}05$

Table 2. PD activity in normal and diabetic rat livers.

Treatment	Specific activity nmol/min/mg protein
Normal	2·7 ± 0.28
Normal + Lipoic acid	4·3 ± 0.19^{a}
Normal + Insulin	4·7 ± 0·42^{a}
Diabetic	1·26 ± 0·29^{a}
Diabetic + Lipoic acid	2·40 ± 0·30^{b}
Diabetic + Insulin	2·66 ± 0·10^{c}

Lipoic acid or insulin was administered intraperitoneally and the animals were sacrificed 60 min later. PD was isolated from the livers as described under Materials and methods

All the determinations are means of 6 animals ± SE

[a] Significantly different from normal at $P < 0{\cdot}01$

[b] Significantly different from diabetic at $P < 0{\cdot}05$

[c] Significantly different from diabetic at $P < 0{\cdot}01$

Effect of lipoic acid on blood sugar and serum insulin levels

The blood sugar and serum insulin levels are shown in table 4. The blood sugar levels decreased significantly in normal rats following lipoic acid administration (60 min after a single intraperitoneal injection of 50 µmol/100 g body wt sodium lipoate in 0·1 ml saline). Although there was a mean reduction in blood sugar values in diabetic rats; 338 and 288 mg/100 ml in saline and lipoic acid injected rats respectively, the reduction was

Table 3. Acylation of DTO catalyzed by rat liver PD.

Thiol	Activity nmol/min/mg protein
CoA	9·25
DTO	5·05

The enzyme was assayed as described under Materials and methods and the activity was expressed as nmol of NAD^+ reduced/min/mg protein. The product of the reaction with DTO was identified by extracting with benzene, separating the products on thin layer chromatography and estimating the acetyl DTO as acetyl hydroxamate

Table 4. Blood sugar and serum insulin levels in normal and diabetic rats.

Treatment	Blood sugar mg/100 ml	Serum insulin μI.U./ml
Normal (20)	110±4·0	59·6±7·9
Normal + Lipoic acid (20)	90±5·3[a]	52·8±8·2[b]
Diabetic (12)	338±54.5	31·6±4·3[c]
Diabetic + Lipoic acid (12)	288±51·4	29·3±3·5[d]

Rats were administered lipoic acid intraperitoneally and sacrificed 1 h later and the blood sugar and serum insulin estimated as described under Materials and methods.
Numbers in parentheses denote the number of animals used for the determination.
All values are means ± SE.
[a] Significantly less than normal blood sugar $P < 0{\cdot}05$.
[b] Not significantly different from normal serum insulin.
[c] Significantly less than normal serum insulin $P < 0{\cdot}01$.
[d] Not significantly different from diabetic serum insulin.

conditions where the blood sugar was estimated from the same rat before and after DTO administration. In the present study, 24 diabetic rats were distributed into 2 groups based on their urinary sugar. Lipoic acid was administered to one group and saline to the other and at the end of 60 min their blood sugar and serum insulin levels were estimated and compared. Since the standard error for blood sugar is large, the reduction is not statistically significant.

The mean serum insulin levels were reduced in diabetic rats as compared to normal etoacetate synthesis (Burch and Wertheim, 1973). From table 5, it can be seen that TO when perfused along with octanoate brought about a 25% reduction in the etoacetate levels in the perfusate. The extent of β-hydroxy butyrate formed was not imated.

Table 5. Acetoacetate levels in liver perfusate.

Treatment	Total (μmol)	Acetoacetate formed μmol/g liver
2 mM octanoate	6·5	0·6
2 mM octanoate + 2 mM DTO	3·4	0·45

Livers from starved normal rats were perfused with octanoate or octanoate and DTO as described under Materials and methods for 1 h. Acetoacetate in the perfusate was estimated according to Walker's procedure (Walker, 1954)

tivation of fatty acids with DTO

tty acyl CoA synthetase from normal rat liver microsomes was used to study the tivation of fatty acids with CoA and DTO. The 1-^{14}C labelled palmitoyl CoA/DTO rmed in the assay were separated by extraction/thin layer chromatography and the dioactivity counted. From table 6, it can be seen that the activity of the enzyme with TO is comparable to that with CoA.

Table 6. Activation of fatty acids with DTO catalyzed by fatty acyl CoA synthetase.

Thiol	CPM incorporated into palmitoyl CoA/DTO	Activity nmol/min/mg protein
CoA	1838	9·8
DTO	1813	9·7

Fatty acyl CoA synthetase was isolated from normal rat liver microsomes and assayed as described under Materials and methods. Palmitoyl CoA formed was estimated by determining the acid soluble counts at the end of the assay. Palmitoyl DTO was estimated by extracting the acidified reaction mixture with ether and separating the free palmitate from palmitoyl DTO on thin layer chromotography using *n* hexane:diethyl ether:acetic acid (80:20:1; v/v) as the solvent system. The band corresponding to palmitoyl DTO was cut out and counted.

iolysis of acetoacetyl DTO

ne thiolase assay was performed by following the reduction in the absorption of the etoacetate functional group spectrophotometrically at 310 nm. Acetoacetyl DTO reactions were reduced; mixed thiolysis of acetoacetyl CoA by a factor of 8 while the thiolysis of acetoacetyl DTO could not be measured (table 7).

Table 7. Thiolysis of acetoacetyl DTO.

Thioester	Thiol	Specific activity*
Acetoacetyl CoA	CoA	5·0
	DTO	0·6
Acetoacetyl DTO	CoA	0·2
	DTO	n.d.**

* Decrease in A_{310} of 0·01/min/mg protein.

** Not detectable.

Thiolase was assayed by the decrease in the 310 nm absorbance (λ max for β-keto ester functional group) indicative of cleavage of the substrate.

Pyruvate carboxylase and acetyl DTO

Oxaloacetate which is the product of the pyruvate carboxylase reaction is measured by coupling it to citrate synthase. Acetyl CoA allosterically activates PC and is also a substrate for the citrate synthase reaction (Utter *et al.*, 1964). Acetyl DTO is also a substrate for citrate synthase (unpublished observation). From table 8 it can be seen that in the presence of acetyl DTO, the activity of PC was negligible while with acetyl CoA the specific activity was 43·8 nmol of citrate formed/min/mg protein. This indicates that acetyl DTO does not activate PC to any significant extent.

Table 8. Effect of acetyl DTO on pyruvate carboxylase.

	ΔA_{412}/min	Specific activity*
Acetyl CoA	0·053	43·82
Acetyl DTO	n.d.**	n.d.**

* nmol of citrate formed/min/mg protein.

** not detectable.

PC was isolated from normal rat liver and assayed by coupling with excess citrate synthase and the activity was determined by estimating the rate of release of free —SH groups using DTNB as described under Materials and methods.

Discussion

Any proposal for the mode of action of lipoic acid must explain its effect in reducing blood sugar, pyruvate and acetoacetate levels and increasing liver glycogen and fat nthesis in diabetic rats. The most obvious explanation would be through stimulation insulin secretion since insulin is known to bring about these changes. Further, lphhydryl containing compounds have been reported to stimulate insulin secretion *in tro* (Chiba, 1969; Haugaard and Haugaard, 1970; Lavis and Williams, 1970). Since TO has two sulphhydryl groups, it is probable that it stimulates insulin secretion also. owever, lipoic acid administration in normal or alloxan diabetic rats did not increase rum insulin levels, thus ruling out insulin as the mediator of the antidiabetic effects of oic acid.

The activity of pyruvate dehydrogenase is reduced in diabetes (Kerbey *et al.*, 1976). he mode of inhibition has been shown to be through phosphorylation of the enzyme. sulin is known to reverse this and thus stimulate the enzyme (Hughes *et al.*, 1980). In earlier study, we had shown that liver lipoic acid levels are reduced in diabetes atraj *et al.*, 1984). In view of this, it is probable that at least a part of the inhibition of D in diabetes could be due to reduced substitution of lipoic acid in the enzyme mplex. It is logical therefore, to expect that PD activity would be stimulated by lipoic id administration in diabetic rats. In normal rats however, it is not possible to explain e observed stimulation of PD by lipoic acid (table 2). It would therefore appear nlikely that the stimulation of PD by lipoic acid in normal or diabetic rats is through creased substitution of the cofactor at the enzyme level alone.

A closer scrutiny of the mode of regulation of PD in diabetes shows that increased cumulation of acetyl CoA in diabetes leads to acylation of enzyme bound lipoic acid Vieland *et al.*, 1971). This in turn stimulates the kinase which inhibits PD through osphorylation. CoA has been shown to overcome this inhibition (Cate and Roche, 83). We have shown that DTO can substitute for CoA in the assay of PD. The roduct of the reaction after extracting from the reaction mixture has been identified as etyl DTO by separating on thin layer chromatography and estimating by the ydroxamate method. We propose that DTO overcomes the inhibition of PD in a anner analogous to CoA. It must be mentioned that O'Connor *et al.* (1982) have own that PD catalyzes the acylation of dihydrolipoamide with acetyl CoA as the acyl onor. The formation of enzyme bound acetyl DTO and the transfer of the acetyl oup to CoA in the reaction pathway of PD have been well established. In this study, e have shown that in the absence of CoA, the acetyl group of the enzyme bound acetyl TO is transferred to DTO with the formation of free acetyl DTO; this is the first emonstration of such a reaction.

While the above accounts for the activation of PD and the resulting lowering of rum pyruvate levels, an important observation from this as well as the previous study atraj *et al.*, 1984) is the lowering of acetoacetate levels by DTO. It is well known that tty acids predominantly furnish the carbon atoms for acetoacetate synthesis (Ohgaku *al.*, 1983). DTO lowers the acetoacetate levels in the perfusate from starved rats when rfused along with octanoate. In order to explain this, we have examined the effect of TO on some of the key steps in fatty acid oxidation.

The terminal four carbon atoms of an even carbon fatty acid during oxidation can ve rise to acetoacetyl CoA as can two molecules of acetyl CoA by head to tail

Fatty acid oxidation starts with the activation of a fatty acid as its CoA thioester. In the present study we have shown that in this reaction DTO can replace CoA effectively. The activated fatty acids are oxidized in successive steps with the formation of acetyl CoA or acetyl DTO at each step through the thiolysis of β-ketoacyl thioester. The reduced rates of thiolysis observed with acetoacetyl DTO as compared to acetoacetyl CoA would indicate that during fatty acid oxidation in the presence of DTO thiolysis is considerably decelerated thus exerting a braking effect on the overall rate of fatty acid oxidation. Further, the head to tail condensation of two acetyl DTO molecules through reversal of thiolase reaction is also slower. These two factors would explain the observed reduction in ketosis following lipoic acid administration.

Increased fatty acid oxidation has also been shown to trigger gluconeogenesis from lactate and alanine in diabetic animals; "the control of pyruvate carboxylation being physiologically the most meaningful interaction between fatty acid oxidation and gluconeogenesis" (Williamson *et al.*, 1969). This has been attributed to the elevation in intramitochondrial acetyl CoA which activates pyruvate carboxylase (Hers and Hue, 1983; McClure and Lardy, 1971). The deceleratory effect of DTO on fatty acid oxidation would also contribute to a reduction in acetyl CoA levels. We have examined the possibility whether acetyl DTO that may be formed from acetyl CoA, would also activate PC in a manner analogous to acetyl CoA. The data presented in table 8 show that acetyl DTO does not stimulate PC, thus contributing to a reduction in gluconeogenesis.

While lipoic acid or DTO can substitute for CoA in all the reactions examined such as formation of acetyl DTO from pyruvate catalyzed by PD, and fatty acyl DTO from fatty acids catalyzed by fatty acyl CoA synthetase, two important differences were observed: (i) the DTO thioester is a very poor substrate for thiolase. In this connection, it must be pointed out that a similar observation has been reported earlier (Drummond and Stern, 1957; Stern and Drummond, 1961); (ii) activation of PC by acetyl DTO was negligible when compared to acetyl CoA. The observation that activation of fatty acids as the DTO thioester proceeds facilely while oxidation of fatty acyl DTO would be expected to be slower, leads one to speculate whether DTO has a dominant or accessory role in fat biosynthesis.

In conclusion we propose that the mode of action of lipoic acid in diabetes is through preferential slowing down of fatty acid oxidation, and gluconeogenesis (since acetyl DTO unlike acetyl CoA does not activate PC) and by overcoming the inhibition of PD by acetyl CoA.

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trontium–calcium discrimination *in vitro* by rat tissues

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Abstract. Uptake of ^{89}Sr and ^{45}Ca by 15 soft tissues of adult rat was studied *in vitro* to assess the extent of discrimination between Sr and Ca. While brain, kidney, placenta and uterus have lower uptake of ^{89}Sr and ^{45}Ca that of diaphragm, lactating mammary gland, skeletal muscle, skin, spleen and testes is higher. Tissues with medium range uptake are heart, small intestine, liver, lung, non-lactating mammary gland and ovary. The 6 tissues displaying discriminating ability, as expressed by ^{89}Sr/^{45}Ca (tissue/medium), in the decreasing order are: small intestine, kidney, lactating mammary gland, placenta, diaphragm and heart. Non-lactating mammary gland and the other tissues did not differentiate between Sr and Ca. The effect of several enzyme inhibitors, compounds influencing Sr–Ca metabolism and other factors was studied in terms of the nature and mechanism of Sr–Ca discrimination.

Keywords. Sr/Ca discrimination *in vitro*; rat tissues.

ntroduction

is known that animal body can discriminate between strontium and calcium and at the extent of such discrimination within the body varies depending on the tissues or e physiological process involved (Comar *et al.*, 1956; Comar and Wasserman, 1964). ut, kidney, placenta and mammary gland display a discrimination against Sr and in vour of Ca of the order of 2–4 (Comar, 1963) as compared with 1·64 by bone Kshirsagar *et al.*, 1966). This Sr/Ca discrimination is manifested during the passage of ese ions across the biological membranes which are under metabolic control. Earlier n active transport mechanism specific for Ca in the intestine of rat has been emonstrated *in vitro* (Schachter *et al.*, 1960). Lengemann (1960) noted a similar echanism in chick bone. Other tissues, however, have not received much attention. In living organism or animal, due to dynamic conditions involved and the simultaneous teraction of tissues such as those mentioned above, the Sr/Ca discrimination by other ssues is likely to be masked. The determination of the discrimination under such ircumstances would be greatly facilitated by studies *in vitro*.

The present investigation was, therefore, undertaken to study *in vitro* the uptake or ccumulation of ^{89}Sr and ^{45}Ca by several tissues.

Materials and methods

Materials

Chemicals: ^{89}Strontium [carrier-free, as $^{89}Sr(NO_3)_2$] and ^{45}calcium (high-specific activity 45 mCi/g, as $^{45}CaCl_2$) were obtained from the Isotope Group, Bhabha Atomic Research Centre, Bombay. The isotopes were used after suitable dilution and adjustment of pH. Sodium arsenate, citrate, fluoroacetate, iodoacetate, malonate and 2-4-dinitrophenol (DNP) were purchased from Sigma Chemical Company, St. Louis, Missouri, USA. Pentobarbitone sodium was obtained from Société des Usires Chimiques Rhone Poulenc, Paris, France. Other reagents and chemicals used were of high grade (analytical) purity.

Animals: Adult female Wistar rats (6–8 months old and weighing about 250–300 g) from the BARC animal colony were used in this investigation. These animals were reared on the colony stock diet (Kshirsagar, 1976a). Normal, pregnant as well as lactating rats were selected. Male rats were used for studies on testis.

Methods

The animals were fasted overnight and killed by stunning. The following tissues were studied: Brain, diaphragm, heart, small intestine, kidney, liver, lung, mammary glands (lactating as well as non-lactating), skeletal muscle (thigh), ovary, placenta, skin, spleen, testis and uterus. These tissues were quickly removed as described earlier (Kshirsagar, 1977a, b). Mammary glands were dissected carefully from the skin, the residual milk, when present, being removed as much as possible by gently pressing under filter paper. Placenta was obtained from rats killed 18–19 days after the onset of pregnancy. The tissues after removal were immediately chilled on ice, cleared of adventitious matter and sliced into thin pieces (about 100 mg in weight) which were bathed in ice-cold medium (0·146 M NaCl–4 mM KCl). The slices were blotted dry on the filter paper and weighed. The uptake of ^{45}Ca and ^{89}Sr in the tissue slices was determined by the method of Schachter *et al.* (1960), with the following modifications. The reaction medium contained 0·15 1M NaCl, 4 mM sodium phosphate adjusted to pH 7·4, 0·02 M glucose, 0·02 μCi per ml each of ^{45}Ca (containing 0·5 μg stable Ca carrier) and ^{89}Sr (carrier-free). Since no extraneous stable Ca^{2+} or Sr^{2+} was added, Sr^{2+}/Ca^{2+} ratio remained constant in the reaction medium in all experiments except the one on the effect of stable Sr (10^{-3} M) on the Sr–Ca discrimination. The flasks containing 5 ml of the medium were gassed with O_2 for 5 min. The tissue slices were added to the flasks and incubated at 37°C for 2 h (a period found to be adequate for the optimum uptake of ^{89}Sr and ^{45}Ca by all tissues as seen from separate studies). The tissues were then removed, blotted dry, weighed and homogenised in 1 ml of 0·1 M Na_2EDTA solution. After centrifugation were run concurrently using tissue slices which were boiled for 5 min in 0·9% NaCl in the case of all the tissues except brain and mammary glands which were, however, denatured by soaking in 10% formalin for 30 min in view of the severe disintegration of these tissues on heat inactivation. The blank variations were within 10%. Uptake of ^{45}Ca or ^{89}Sr by tissue was expressed as the final concentration of ^{45}Ca or ^{89}Sr in tissue (cpm/g). The Sr-Ca discrimination was expressed as the observed ratio (OR) tissue-medium (as proposed by Comar *et al.*, 1956).

$$\text{OR (tissue-medium)} = \frac{{}^{89}\text{Sr}/{}^{45}\text{Ca tissue (final concentration)}}{{}^{89}\text{Sr}/{}^{45}\text{Ca medium (final concentration)}}.$$

It may be mentioned here that a low value of OR indicates high discrimination against Sr and in favour of Ca and *vice versa.*

Results

The tissue uptake of ^{89}Sr and ^{45}Ca (cpm/g) reaches a maximum at 2 h as illustrated typically in table 1. The uptake of ^{89}Sr and ^{45}Ca of various tissues, the ratio of concentration in tissue (T)/medium (M) of ^{89}Sr as well as ^{45}Ca and the Sr–Ca discrimination ratio (OR) are described in table 2.

^{89}Sr and ^{45}Ca uptake

In general, the tissues fall in three categories depending upon the extent of uptake: (i) those with comparatively lower uptake, 10,000–20,000 cpm/g of both ^{89}Sr and ^{45}Ca, like brain, kidney, placenta, and uterus; (ii) tissues with higher uptake of 40,000–50,000 cpm/g, *e.g.* diaphragm, lactating mammary gland, skeletal muscle, skin, spleen and testes; (iii) the remaining tissues with uptake of medium range.

Table 1. Uptake of ^{89}Sr and ^{45}Ca by tissues at different periods of incubation.

		Uptake (cpm/g)			
		Period (h)			
Tissue	Isotope	0·5	1	2	4
Kidney	^{89}Sr	4170	9220	10172	10488
	^{45}Ca	7067	14908	15820	15183
Small intestine	^{89}Sr	7292	14588	14818	14821
	^{45}Ca	13542	27777	23806	26349
Diaphragm	^{89}Sr	21782	40200	41277	39715
	^{45}Ca	29418	49350	49660	47819

Activity of medium before incubation = about 10,000 cpm/ml each of ^{89}Sr and ^{45}Ca.

Table 2. Sr–Ca discrimination by various tissues.

	Activity of tissue (T) cpm/g		Activity of tissue T / Activity of medium M		Observed ratio
Tissue	^{89}Sr	^{45}Ca	^{89}Sr	^{45}Ca	OR″
Brain	9620 ± 737	10600 ± 1239	0·98 ± 0·11	1·04 ± 0·11	0·94 ± 0·12
Diaphragm	40920 ± 2302	49230 ± 1221	5·09 ± 0·81	7·00 ± 0·35	0·73 ± 0·10
Heart	20701 ± 1062	27294 ± 1830	2·88 ± 0·49	3·50 ± 0·44	0·75 ± 0·04
Small intestine	16947 ± 1061	31618 ± 1502	2·14 ± 0·09	4·87 ± 0·19	0·44 ± 0·04
Kidney	9553 ± 687	13888 ± 647	1·03 ± 0·06	1·69 ± 0·14	0·61 ± 0·07
Liver	24202 ± 3059	25147 ± 2272	3·09 ± 0·31	3·01 ± 0·28	1·03 ± 0·06
Lung	22014 ± 1104	24241 ± 1914	2·43 ± 0·18	3·06 ± 0·28	0·89 ± 0·08
Mammary gland non-lactating	31815 ± 2296	30482 ± 2802	3·54 ± 0·33	3·51 ± 0·34	1·00 ± 0·09
Mammary gland lactating	36680 ± 3001	51085 ± 4019	4·72 ± 0·36	7·77 ± 0·69	0·61 ± 0·05
Skeletal muscle	42675 ± 2890	44643 ± 2635	6·28 ± 0·25	6·80 ± 0·38	0·92 ± 0·05
Ovary	21807 ± 2274	22483 ± 2095	2·68 ± 0·30	3·06 ± 0·30	0·88 ± 0·04
Placenta	12139 ± 1968	16570 ± 1257	1·34 ± 0·17	2·94 ± 0·15	0·62 ± 0·09
Skin	39156 ± 2445	38941 ± 2203	4·83 ± 0·66	4·67 ± 0·75	1·02 ± 0·11
Spleen	37831 ± 2492	40211 ± 2101	5·29 ± 0·89	5·11 ± 0·82	1·04 ± 0·09
Testis	50554 ± 4008	48142 ± 4277	6·58 ± 0·58	6·36 ± 0·70	1·03 ± 0·09
Uterus	15994 ± 1070	13637 ± 988	1·81 ± 0·09	1·75 ± 0·06	1·03 ± 0·04

$$\mathrm{OR}'' = \frac{^{89}\mathrm{Sr}/^{45}\mathrm{Ca}\ \text{tissue}}{^{89}\mathrm{Sr}/^{45}\mathrm{Ca}\ \text{medium after incubation}}.$$

Activity of medium before incubation = about 10,000 cpm/ml each of ^{89}Sr and ^{45}Ca; $^{89}\mathrm{Sr}/^{45}\mathrm{Ca} = 1$.
Mean ± S.E.M. observation on 6 adult rats (6–8 months old).

Sr–Ca discrimination (OR)

Only 6 of the 15 tissues studied manifested Sr–Ca discrimination. Of these, small intestine evinced it to the highest degree (OR—0·44) followed by kidney, lactating mammary gland and placenta (all with OR—0·61). Diaphragm and heart handled Sr and Ca differentially to a moderate extent, their OR being about 0·74. The non-lactating mammary gland and other tissues failed to display any selective power.

Results of the experiments involving incubation with N_2 (in place of O_2) and at 4°C (instead of 37°C) as well as the effect of the metabolic inhibitor DNP (10^{-3} M) depict the involvement of metabolic process in the Sr–Ca discrimination (table 3).

Although the effect on OR of various metabolic inhibitors and compounds influencing Sr and Ca metabolism was studied at 10^{-6}–10^{-3} M levels, only the typical results (OR) of a few compounds at 10^{-3} M level have been given in table 4. The salient changes in the uptake of ^{89}Sr and ^{45}Ca due to DNP, citrate and lactose, the three representative compounds showing notable effects, have been described in table 3 to illustrate the results. Sr–Ca discrimination was decreased to widely varying extent due to metabolic inhibitors, in general, and in many instances to lactose and lysine. Citrate able 3. Effect of some factors on ^{89}Sr–^{45}Ca uptake and discrimination (OR″).

	Isotope	OR″	Small intestine	Kidney	Mammary gland (lactating)	Placenta	Diaphragm	Heart
	^{89}Sr		16155	10172	38160	12800	34715	17352
$_2$–37°C	^{45}Ca		32877	14318	51600	16874	40280	23030
		OR	0·40	0·63	0·62	0·64	0·75	0·74
	^{89}Sr		4292	2164	6876	2640	6940	4170
$_2$–37°C	^{45}Ca		3864	2224	7592	2832	6444	3684
		OR	1·02	0·97	0·91	0·93	1·04	1·01
	^{89}Sr		2908	1668	5088	2136	4164	2776
$_2$–4°C	^{45}Ca		2968	1844	4584	2220	4832	2764
		OR	0·98	0·94	1·04	1·00	0·96	0·97
	^{89}Sr		11511	10760	19710	13240	21782	11875
NP (10^{-3} M)	^{45}Ca		13488	11800	20315	13040	21409	12131
		OR	0·88	0·90	0·94	1·00	1·01	0·97
	^{89}Sr		18417	9220	34950	11457	35200	20710
itrate (10^{-3} M)	^{45}Ca		55562	10922	77250	19200	56500	34210
		OR	0·30	0·85	0·41	0·58	0·60	0·62
	^{89}Sr		26976	11202	59260	13244	36277	15368
actose (10^{-3} M)	^{45}Ca		38513	17646	62940	17190	44660	22105
		OR	0·71	0·62	0·93	0·65	0·75	0·72

$$\text{R} = \frac{^{89}\text{Sr}/^{45}\text{Ca tissue}}{^{89}\text{Sr}/^{45}\text{Ca medium after incubation}}.$$

ctivity of medium before incubation = about 10,000 cpm/ml each of ^{89}Sr and ^{45}Ca; ^{89}Sr/^{45}Ca = 1·00.
ptake-cpm/g.
ean observations on 4 adult rats (6–8 months old). Standard error was about ±6% of the mean.

iscussion

he high uptake of ^{45}Ca and ^{89}Sr by diaphragm (composed of fibres and muscles) and keletal muscle is noteworthy in view of the active accumulation of Ca^{2+} by the white uscle of rabbit (Ebashi and Lipman, 1962) and by some cellular components of uscles (Martonosi *et al.*, 1971; Shane, 1963). ^{89}Sr and ^{45}Ca accumulation by testes is rikingly high—2–3 times—as compared with that of ovary and uterus. The increase in ptake, 15% for ^{89}Sr and 70% for ^{45}Ca, in the lactating as compared to the non-ctating mammary gland is of interest since Ca is known to be concentrated at least 10 ld or more during passage from blood to milk (Twardock, 1963). It is pertinent to ote that non-lactating mammary gland failed to select between Sr and Ca unlike its ctating counterpart. The discrimination is apparently attributable to the functional etabolic) changes set in during the process of lactation.

Table 4. Effect of different compounds on Sr–Ca discrimination (OR[a]).

Compound	Small intestine	Kidney	Mammary gland (lactating)	Placenta	Diaphragm	Heart
Control	0·37	0·65	0·63	0·62	0·73	0·75
Arsenate	1·00	0·96	0·85	0·84	0·98	0·94
Cyanide	0·86	0·81	0·87	0·73	0·90	0·88
Dinitro phenol	0·88	0·90	0·94	1·00	1·01	0·97
Fluoride	0·51	0·68	0·77	0·80	0·81	0·85
Fluoroacetate	0·98	0·85	0·86	0·70	0·93	0·90
Mercuric chloride	0·58	0·73	0·92	0·85	0·80	0·87
Citrate	0·30	0·85	0·41	0·58	0·60	0·62
Lactose	0·71	0·62	0·93	0·65	0·75	0·72
Lysine	0·55	0·67	1·12	0·61	0·78	0·71
Strontium chloride	0·20	0·68	0·67	0·67	0·61	0·65

[a] $\mathrm{OR} = \dfrac{{}^{89}\mathrm{Sr}/{}^{45}\mathrm{Ca}\ \text{tissue}}{{}^{89}\mathrm{Sr}/{}^{45}\mathrm{Ca}\ \text{medium after incubation}}.$

Concentration of compounds = 10^{-3} M.
Activity of medium before incubation = about 10,000 cpm/ml each of ^{89}Sr and ^{45}Ca; ^{89}Sr and ^{45}Ca = 1·00.
Mean observations on 4 adult rats (6–8 months old).
Standard error was about ±5–10% of the mean.

albeit to a limited extent. The OR values reported here for the first four tissues compare well with those reported in the literature (Comar, 1963; Comar and Wasserman, 1964).

Tissue Sr–Ca discrimination was severely affected by metabolic inhibitors, particularly arsenate, cyanide, dinitro phenol and fluoroacetate (OR being about 1) (table 4) which agrees well with the active transport hypothesis (Schachter *et al.*, 1960; Taylor, 1964). The adverse effect, as seen by the increase in OR, of lactose and lysine on the discrimination by small intestine and lactating mammary gland tends to support the 'metabolic block' hypothesis for Ca absorption (Wasserman, 1964; Taylor, 1967). Lactose and lysine act perhaps by modifying (possibly facilitating) diffusion and hence absorption of Ca and more particularly Sr. Absorption is increased by about 40–70% for Sr and 10–20% for Ca due to lactose and lysine in small intestine and lactating mammary gland. The effect on the latter tissue is of particular significance since both the compounds are important constituents of milk diet. Incidently milk proteins have been shown to display greater affinity for Sr than Ca (Twardock *et al.*, 1960).

Citrate (a chelating agent possibly involved in the Ca-transfer mechanism in the cell) enhanced the differential treatment in the small intestine, lactating mammary gland, diaphragm and heart. This is a result of the better absorption of Ca due to its higher stability constant than that of Sr with citrate (Schubert and Lindenbaum, 1952). The decrease in the renal discrimination due to citrate is, however, intriguing. Interestingly similar decrease has also been observed by Dells Rosa *et al.* (1961) and Walser and Robinson (1963) who demonstrated a differential reabsorption *i.e.* more of Ca than of f stable Sr on the intestinal discrimination is due to isotopic dilution, the carrier Sr ompeting with ^{89}Sr for sites of absorption *e.g.* intestinal Ca binding protein which inds Sr also (Wasserman *et al.*, 1968; Menczel and Mor, 1972).

The T/M ratios for ^{89}Sr and ^{45}Ca (table 2) reveal active transport for ^{89}Sr also, hough on a lower scale as compared with ^{45}Ca, in many tissues of rats reared on a diet nown to sustain normal Ca and P levels in tissues (Kshirsagar, 1976b). This is in greement with the observation of active transport for small intestine by Kimberg *et al.* 1960) and for human erythrocyte ghost by Olson and Cazart (1969) while differing rom that of Wasserman (1960). This study thus shows that Sr–Ca discrimination may nvolve differences in active transport as well as facilitated diffusion of Ca and Sr Schachter *et al.*, 1961).

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n approach to improve methanogenesis through the use of mixed ultures isolated from biogas digester

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Abstract. Biogas production has been shown to be inhibited by branched chain fatty acids (isobutyric, isovaleric) produced in the digester by cellulolytic organisms. Performance of these mixed cellulolytic cultures isolated at 25°C (C_{25}) and at 35° (C_{35}) in a batch digester using cattle manure confirmed that C_{35}, which forms mainly straight chain fatty acids from cellulose was more suitable for use as an inoculum than C_{25} which formed predominantly branched chain fatty acids. Reconstitution of cellulolytic culture C_{35} and mixed methanogens M_{35} almost doubled both the amount and rate of methane production. Cellulolytic culture was useful in pretreatment of water hyacinth prior to its use as a substrate for methane generation. A method for preservation and transportation of mixed cellulolytic culture for use as an inoculum in the digester is described.

Keywords. Improved methanogenesis; biogas; mixed cultures for biogas.

ntroduction

naerobic digestion of livestock wastes for production of biogas is carried out by icroorganisms in three different stages. During the first stage, solids are solubilised by ction of microorganisms which convert cellulosic materials to sugar, volatile fatty cids (VFA), hydrogen and carbon dioxide. In the second stage, these products are urther converted to acetic acid, a process considered to be thermodynamically the least avourable and functions well only in combination with the third stage which convert cetate to methane and carbon dioxide (Klass, 1984; Daniels, 1984). However, in an verall complex process containing large amounts of lignocellulosic wastes, cellulose ydrolysis may be limiting. Current concepts in reactor designs for methane roduction are essentially directed towards overcoming these limitations. The process f anaerobic digestion is, therefore, carried out in a two phase system. In the first phase, gnocellulosic material is digested to soluble products which are fed to a second reactor n which the last two stages are combined to produce methane and carbon dioxide Khan *et al.*, 1983; Wandrey and Aivasidis, 1984; Chen *et al.*, 1980). A prime requisite in uch a process is the development of suitable mixed cultures to carry out these steps fficiently.

In our earlier studies we had described isolation of two types of mixed cultures: i) cellulolytic organisms which converted cellulosics to VFA and (ii) methanogens

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bbreviations used: VFA, Volatile fatty acids; GLC, gas–liquid chromotography.

which converted the VFA to methane and carbon dioxide (Kalle and Menon, 1984). It was also demonstrated that cellulolytic cultures selected at 25°C (C_{25}) produced primarily branched chain fatty acids (about 80–85% of VFA) which completely inhibited methane production by methanogens. The mixed cellulolytic cultures selected at 35°C (C_{25}), on the other hand, produced comparatively less amount of these acids (about 40–45% of VFA). In this paper, we present the results of the studies on relative performance of these cultures in a biogas digester with livestock manure and plant material (water hyacinth) as substrates.

Materials and methods

Microbiological techniques

Enrichment of mixed cellulolytic cultures (C_{25}, C_{35}) and methanogens (M_{35}) used in these studies, their source, methods of cultivation, media and anaerobic culture techniques employed have been described in detail in an earlier paper (Kalle and Menon, 1984). Microbial population in mixed culture was adjusted to about 2–4 $\times 10^8$ per ml before inoculation into the digester.

Studies with cattle manure as substrates

All experiments were carried out in duplicate in glass fabricated batch digesters containing 1·6 kg of 50% slurry (8% solids) at 35°C to which 0·4% urea was added. In the experiment to study the effect of mixed cellulolytic cultures on the digestion of cattle manure and on methane production, the digesters were charged with presterilised manure (15 psi, 15 min.) and inoculated with either C_{25} or C_{35} at 10% level. On the 28th and 30th day, when VFA production was stabilised, the digester was replenished with 80 g (5%) of fresh cattle manure (50% slurry) as a source of inoculum for methanogens present normally (figure 1). The digestion was continued upto 60 days. In the experiment to test the performance of both cellulolytic (C_{35}) and methanogenic (M_{35}) mixed microflora, sterilised manure was inoculated with equal quantities of mixed cultures at 10% level in the digester. As a control, another digester was simultaneously run with fresh (unsterilised) manure with naturally resident microflora (figure 2). The VFA, total gas and methane were monitored throughout the period of experiment. In both the digesters, inoculum was replenished on the 10th and 15th day.

Studies with water hyacinth as a substrate

Predigestion of plant material: Water hyacinth was chopped and dispensed into 24 plastic pouches (200 g per packet). The total solid content was maintained at 5% in all the cases. Prior to sealing, 12 packets were inoculated with cellulolytic culture to give approximately 10^7 cells/g of slurry. Remaining 12 served as controls with indigenous microflora for comparison. The packets were sealed leaving a marginal air space

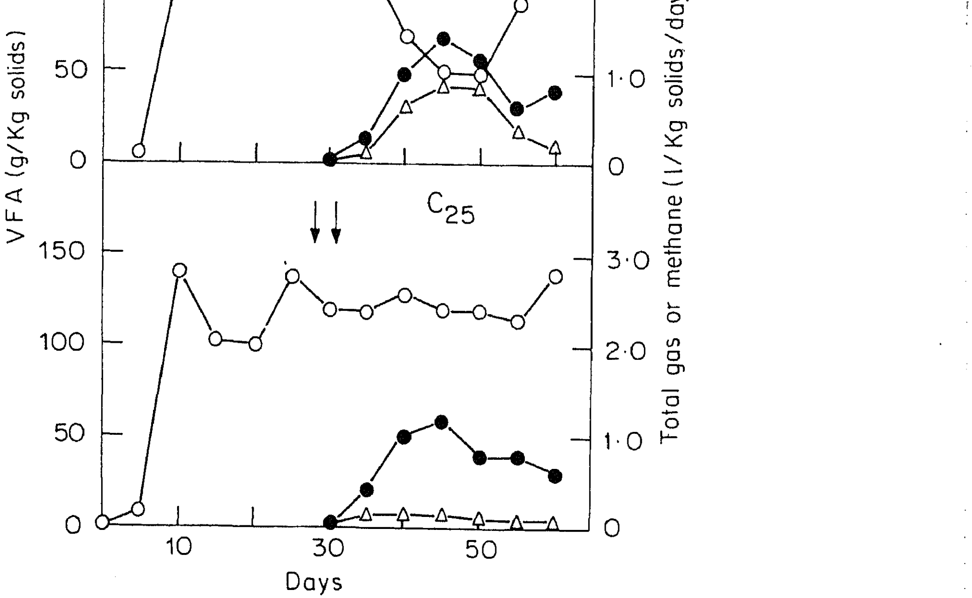

Figure 1. Production of VFA by mixed cultures C_{35} and C_{25} from sterilized cattle manure and its effect on methane production. Fresh manure was added at a rate of 5% solids in the digester as a source of methanogen on the 28th and 30th day (O), VFA; (●), total gas; (Δ), methane.

f remaining packets were pooled and analysed for volatile solids, crude fibre, ash, itrogen and VFA. The VFA recovered were further analysed for individual fatty acids n gas–liquid chromatography (GLC).

Iethane production from water hyacinth: Freshly harvested water hyacinth was hopped into small pieces and made into a slurry (1:3) with water to give approximately % solids. The slurry (1·6 kg) was fed to two of the batch laboratory digesters (capacity l.) which served as control. The remaining 4 digesters contained 0·8 kg of this slurry, ivided into two sets, one mixed with 0·8 kg of plant material digested with resident icroflora and the other with material digested with mixed cellulolytic culture C_{35} as escribed above. The gas was collected over a column of 0·1 N HCl from which it was ontinuously tapped for GLC analysis for methane and carbon dioxide (Kalle and Ienon, 1984). All digesters were incubated for 20 days when further gas production opped.

reservation of cellulolytic culture C_{35}: Garden soil was dried overnight in an oven 10°C), ground and finely sieved to attain a free flowing powder. The soil (80 g) was

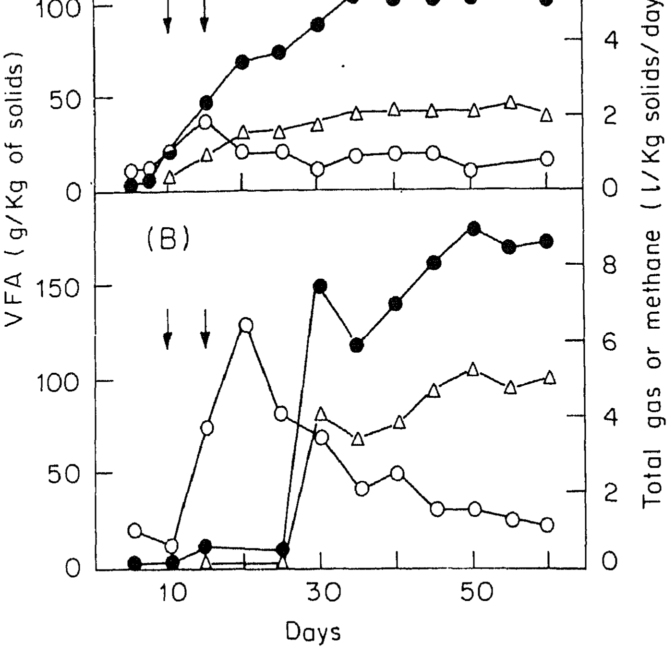

Figure 2. Production of VFA, total gas and methane in batch digester. (A) Fresh unsterilised manure was used as a substrate and a source of inoculum. Digester was replenished with additional manure (5% solids) on the 10th and 15th day. (B) Sterilized manure was used as a substrate and inoculum comprised of C_{35} and M_{35}. The digester was replenished with 5% of inoculum on the 10th and 15th day. (O), VFA; (●), total gas; (Δ), methane.

mixed with cellulose powder (20 g) and distributed 2 g each into vials. Mixed culture C_{35} grown in Hobson's medium (Hobson, 1969) was added to the sterilised soil in the vial to give approximately 10^8 cells/g. Vials were evacuated and flushed with nitrogen. They were sealed under nitrogen. The cultures retained 80% of the viability after 3 months.

Analytical methods: Total volatile solids, crude fibre, ash, total nitrogen and dry matter were determined according to the methods described in AOAC (1975). The VFA from the digester was recovered by steam distillation and estimated as per the method of Rand *et al.* (1979). After acidification and extraction with ether, it was analysed by GLC for individual fatty acids using SP 1200 column at 110°C. The analytical method used for monitoring methane in the gas has been described in detail (Kalle and Menon, 1984).

Performance of mixed cellulolytic cultures C_{25} and C_{35} in a normal batch digester was quite predictable (figure 1). Both the cultures accumulated VFA rapidly. There was no methane formation because the mixed cultures have been purified free of methanogens and digestions were carried out with presterilised manure. When fresh unsterilised cattle manure, which served as a fresh inoculum for naturally occurring methanogens, was added, both gas (CO_2, H_2) and methane production resumed. This was accompanied by a rapid drop in VFA concentration in the case of C_{35} but not in the case of C_{25} which accumulated predominantly isobutyric and isovaleric acids. These were not metabolised and inhibited methane formation.

Data presented in figure 2 shows the combined performance of cellulolytic organisms (C_{35}) and methanogen (M_{35}). The VFA and methane formation with fresh unsterilised manure was also monitored for comparison. With this control there was a steady increase in methane formation and the VFA in the digester were maintained at more or less a constant level. On the other hand, in the system operating with mixed cultures, there was a rapid increase in VFA prior to the synthesis of methane, which resumed only after the VFA levels reached a maximum. Furthermore, methane synthesis was accompanied by a rapid drop in VFA content in the digester, suggesting its rapid utilisation by methanogens for biogas production. The performance of the digester containing the mixed cultures was superior both in terms of rate and amount of methane formed.

Performance of the mixed cellulolytic culture (C_{35}) in digesting plant material

Water hyacinth is a vascular plant with phenomenal growth rates and is considered to be a serious aquatic weed problem. Today, however, its application in various fields has been recognised. Due to its high protein and mineral content, it holds promise as a food and feed supplement (Boyd, 1970; Taylor and Robbins, 1968). It can also be used in control of water pollution (Wolverton, 1975). In a country like India, its potential use in a biogas system is immense. However, overall yields of methane with water hyacinth as a substrate are generally not satisfactory. A possible solution is to predigest the plant material (composting), particularly cellulosic substrates with a mixed culture prior to adding it to the digester.

Table 1 presents analytical data on predigestion of plant material with normal resident microflora (composting) and that incubated with mixed culture C_{35}, which was definitely superior both in terms of VFA formed (12·5 %) and overall digestion efficiency as indicated by reduction in crude fibre by about 67 %. There was no significant difference in fatty acid composition of the digested material. Both showed relatively higher proportion of propionic and branched chain fatty acids. Better digestion in plant material incubated with C_{35} makes it a more acceptable substrate for methane production as indicated by the data presented in table 2. Both the total gas and methane formed in this case were twice that produced by undigested material and 60 % more than by composted plant material. There is, therefore, a definite advantage of

	Water hyacinth		
		Digested (one week)	
Composition	Fresh	Resident microflora Per cent of dry matter*	Mixed microflora C_{35}
Total volatile matter	83·3	86·0	89·8
Crude fibre	34·0	16·5	11·2
Ash	16·7	16·0	15·8
Total nitrogen	2·82	2·5	2·6
VFA	0·46	3·29	12·5
Fatty acids		*Per cent volatile fatty acids*	
Acetic	47·6	9·93	5·7
Propionic	14·7	55·08	64·09
Isobutyric	3·7	6·84	3·60
Butyric	30·5	6·0	1·6
Isovaleric	1·5	6·94	6·3
Valeric	2·0	4·54	3·0
Other acids**		10·67	15·71
Branched chain fatty acids (%)	5·2	13·78	9·9

* Water hyacinth biomass contained 85% moisture and 15% total solids.

** A number of minor aliphatic acid peaks were observed, but not identified.

Table 2. Methane production with water hyacinth as a substrate.

	Water hyacinth		
		Predigested (1 week)	
Composition	Undigested	Resident microflora	Mixed microflora C_{35}
Initial solids			
Wet weight (g)	400	400	400
Dry weight (g)	48	48	48
Moisture (g)	352	352	352
Water added (l.)	1·2	1·2	1·2
Total gas (l./kg dry wt.)	198	235	350
Methane (l./kg dry wt.)	93	127	203
	88	133	234
(% of gas)	47	54	58

Studies were undertaken to develop a scheme for delivery of active cellulolytic culture C$_{35}$) from laboratory to the farmer for use as inoculum in the biogas digester. Problems associated with achieving this objective would be:

i) Preservation and transport of anaerobic cultures, and
ii) scale up of inoculum on site, *i.e.*, on farm.

The cultures involved with biogas system are strictly anaerobic. It was, therefore, ssential to preserve the enriched cultures so as to retain maximum survival. Soil was onsidered a good carrier system for cellulolytic cultures. Cellulose was added to the ystem as the cultures under study were found to adhere to cellulose particles, thereby ncreasing their viability. Soil cultures were sealed under nitrogen to provide anaerobic onditions during storage. In such a system, mixed cellulolytic culture C_{35} retained upto 80% of initial viability at ambient temperatures (30–35°C) at the end of 3 months torage and at refrigerated temperatures upto one year storage. Inoculum is easily built up by growing the culture with plant material in plastic pouches as used in these studies.

Discussion

Attempts have been made to reconstruct model systems using pure strains of *Acetivibria cellulolyticus* and *Methanosarcina barkerii* for conversion of cellulose to methane (Khan and Laube, 1980). In practice, however, use of pure strains is not advisable, since microbial systems are confronted with a multitude of substrates in the form of wastes whether it is derived from livestock manure or plant material. In the operation of any biogas digester, initial work-up procedures are essentially designed to select out cultures suitable for biogas production (Claussen *et al.*, 1981; Subramaniam, 1977). However, in such a system, it is difficult to foresee the performance of individual group of organisms (Ghose and Klaas, 1978; Daniels, 1984) or predict optimal reaction rates (Klass, 1984).

A major advantage of using partially purified mixed cultures lies in the fact that it is possible to eliminate from the system those organisms which produce products nhibiting methane synthesis. This is illustrated by the performance of the two cellulolytic cultures reported here. Culture C_{25} produces predominantly branched chain fatty acids inhibitory to methane formation while these are not formed by the culture C_{35} (figure 1). This was primarily responsible for the better performance of the atter both in terms of cellulose digestibility and in providing suitable precursors (straight chain fatty acids) to methanogens (figure 2). A rapid build up of VFA before the onset of methane synthesis could be, because of the time taken by methanogens to establish themselves in the reactor. To reduce such a time lag, there was need to use a larger inoculum in the batch digester. It must be noted that in an operating biogas digester, methanogens are firmly established only when the O/R potential of the system drops below 350 mv (Taylor, 1975; Smith *et al.*, 1980) and efficiency of the system is resumption of methane production is perhaps a reflection of the time taken to establish these conditions.

The best application of the mixed cellulolytic cultures is in treatment of plant material before adding it to the digester. With the water hyacinth, predigested with C_{35}, the methane yields were far superior to that obtained with the undigested material or that digested with normal associative microflora. Since composting of farm wastes is a conventional procedure and a farmer is conversant with the technique, adaptation and further scale up of this process is possible on site. The plant waste thus composted can serve as a convenient means for the introduction of the cellulolytic culture to the biogas digester. However, the problem associated with this procedure is to preserve and transport the inoculum from the laboratory to the farmer. We have described here an easy method of preservation which provides a shelf life upto 3 months at ambient storage conditions. Providing stability to methanogens is more difficult because of the exacting conditions required for their survival.

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upra-molecular organization of adenylate cyclase in a euroblastoma × glioma hybrid

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Abstract. Studies on the thermal inactivation of adenylate cyclase from neuroblastoma × glioma hybrid cells have been carried out. Inactivation curves show marked deviation from first-order kinetics, and as a first approximation can be adequately described as a sum of two negative exponentials. Half-lives of the rapidly decaying component have been estimated to be 5, 3·4, 1·2 and 0·5 min at 37, 40, 44 and 48°C, respectively. The corresponding values for the slow-decaying component are found to be 90, 30, 11 and 5 min. Plausible inactivation pathways responsible for multi-exponential decay curves are discussed. Kinetic curves describing fractional loss of stimulatory response of adenylate cyclase to prostaglandin E_1 are shifted downwards with reference to basal activity. In contrast, an upward shift is observed for the inhibitory response of the enzyme to etorphine. A quantitative analysis of the inactivation curves for prostaglandin and etorphine-responsiveness has led to definitive predictions regarding the heat-sensitivity of the 'hypothetical' temperature-labile component responsible for the observed shifts.

Keywords. Adenylate cyclase; opiates; neuroblastoma × glioma hybrid cells; receptors.

ntroduction

Modulation of adenylate cyclase activity by the effector molecules is brought about by pecific receptor proteins (Robinson *et al.*, 1971). It is known that adenylate cyclase onsists of at least three distinct entities: the hormone receptor, a guanine nucleotide inding protein (N) and a catalytic unit. Two N proteins mediating the hormone nduced stimulation of adenylate cyclase (N_s) and a second mediating the inhibition of ormones (N_i) are known (Cooper, 1982). All the three entities in the 'native' state are ssociated with particulate fractions, embedded in membrane matrix. An understanding of the spatial and temporal organization of adenylate cyclase-receptor complex is rucial to the development of a unified conceptual framework for the central role scribed to adenosine cyclic 3′,5′-monophosphate (cyclic AMP) in the regulation of iverse types of metabolic processes.

Temperature-inactivation (or heat-sensitivity) of enzyme activity, in essence, represents one of the many parameters used in the studies of protein denaturation (Joly, 965; Johnson *et al.*, 1954). The technique, albeit the difficulties encountered in the nalysis of inactivation process at physico-chemical level, has proved to be a useful tool

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in the studies of conformational transitions in enzyme molecules (Nathan, 1973). The adenylate cyclase in neuroblastoma × glioma cells has been shown to elicit both stimulatory and inhibitory response to a variety of effector molecules such as prostaglandin E_1 (PGE_1) and etorphine as reported by Sharma *et al.* (1975a, b, 1977) and Sharma (1976), and is thus particularly suited for an investigation of the nature of enzyme-receptor complex. In this paper some of the observations on the heat-sensitivity of PGE_1 and etorphine-modulated adenylate cyclase activity obtained from neuroblastoma × glioma hybrid cells are reported.

Materials and methods

Materials

The following chemicals were obtained from commercial sources: Cyclic-G-[^3H]-AMP (22·1 ci/mmol), New England Nuclear; α-^{32}P ATP (6-10 ci/mmol) ICN; Creatine Kinase (155 U/mg of protein) and phosphocreatine, Sigma Chemical Co., St. Louis, Missouri, USA. Naloxone-HCl was a gift from Endo. Labs., Etorphine from Dr. R. Willette of NIDA, PGE_1 from Dr. John Pike of Upjohn Co. and 4-(3-butoxy-4-methoxybenzyl)-2-imidazolidinone ($R_0$20-1724) from Hoffman La-Roche.

Cells

Neuroblastoma × glioma hybrid cell line (NG 108-15) derived from fusion of mouse neuroblastoma clone N18TG-2 and rat glioma clone C6BU-1 was obtained from Dr. Marshall Nirenberg, N.I.H., USA. Cells were grown as described earlier (Sharma *et al.*, 1975a).

Enzyme preparation

In experiments using whole homogenate as a source of adenylate cyclase, confluent cells grown as monolayer in 100 mm plastic petri dishes were washed twice with 50 ml of phosphate-buffered saline (pH 7·4) containing NaCl (137 mM), KCl (5·4 mM), Na_2HPO_4 (0·17 mM), KH_2PO_4 (0·22 mM), $CaCl_2$ (1·8 mM) and glucose (25 mM). Cells were harvested by washing monolayers twice with 50 ml saline of pH 6·5 without calcium, composition of this solution being otherwise similar to that described above. The cell pellets obtained after centrifugation at 500 *g* for 5 min at room temperature were homogenized in 0·32 M sucrose, 10 mM Tris-HCl buffer (pH 7·5) in a homogenizer with Teflon pestle at 0°C. The volume of the homogenizing medium was adjusted so as to obtain a concentration of 10–15 mg protein per ml.

In some experiments, where particulate fractions have been used as a source of enzyme, the homogenate in 0·32 M sucrose, 10 mM Tris-HCl pH 7·5 (homogenizing medium) was centrifuged at 1,000 *g* for 10 min to sediment nuclei, unbroken cells etc. The supernatant fraction was centrifuged at 17,000 *g* for 30 min. The 17,000 *g* pellet was washed, resuspended in homogenizing medium and used as the source of enzyme.

defined by the homogenizing medium. The enzyme preparation (initially at 0°C) was transferred to waterbath at the inactivation temperature. A period of 30 sec was routinely allowed for temperature-equilibration before taking out '0' time sample. At appropriate time-intervals thereafter samples (100–200 μg protein) were removed and adenylate cyclase activity was determined (Salomon *et al.*, 1974). The reaction mixture in each assay tube contained 35 mM Tris-HCl (pH 8·0), 5 mM $MgCl_2$, 160 mM sucrose, 20 mM creatine phosphate, 10 U of creatine kinase, 1 mM cyclic AMP, 0·25 mM R_0 20-1724 and 1 mM α-^{32}P ATP ($6–8 \times 10^6$ CPM) in a final volume of 100 μl. The mixture contained ethanol at a final concentration of 0·2% (used in dissolving R_0 20-1724). The reaction was initiated by the addition of enzyme at 37°C and was carried out at the same temperature. At the end of incubation period (5 min) the reaction was terminated by adding trichloroacetic acid at 0°C to a final concentration of 5%. Methods for separation of cyclic AMP and determination of its radioactivity have been described elsewhere (Sharma *et al.*, 1975a).

Results

Figure 1A represents decay curves for basal adenylate cyclase activity in which enzyme activity expressed as a fraction of that at '0' time is plotted against time of exposure to various temperatures on a semi-logarithmic scale. The enzyme activity, as seen from the

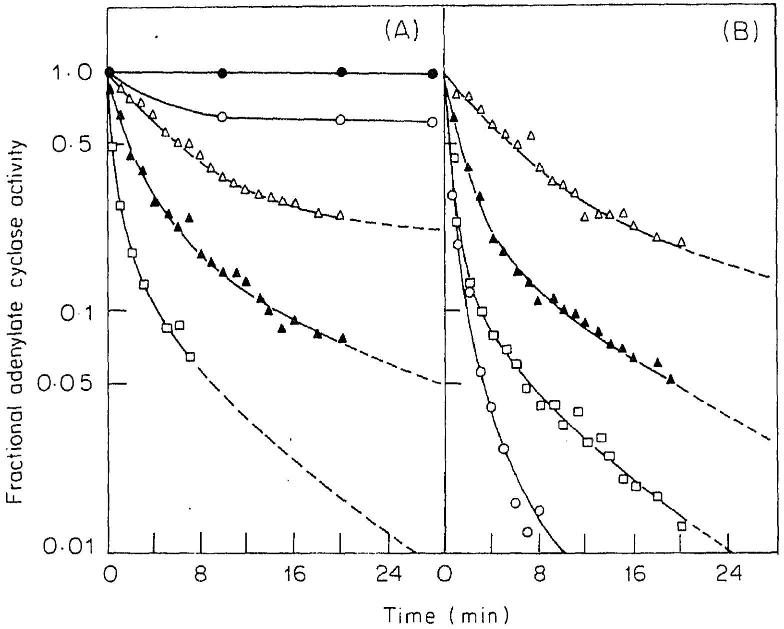
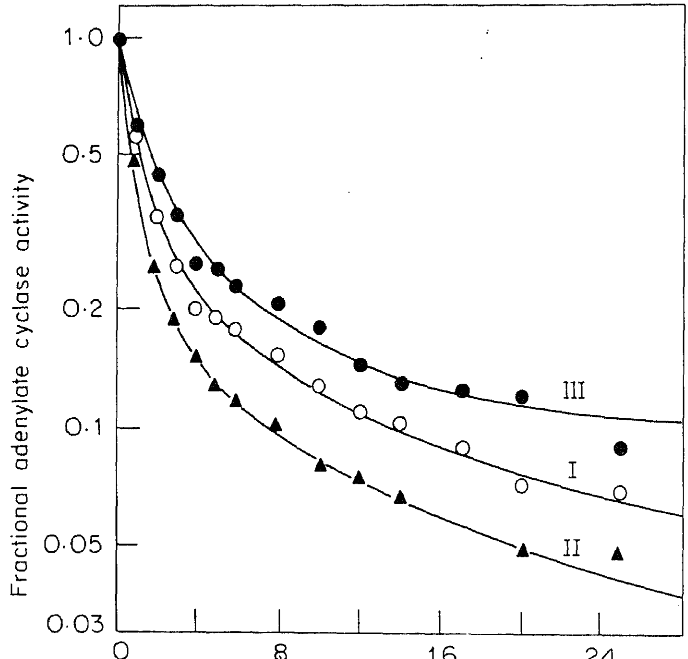


Figure 1. Time-course of heat-inactivation of adenylate cyclase activity in the homogenate

top horizontal line in figure 1A, is quite stable at 0°C under our experimental conditions. It is noticed that even at 37°C about 40% of the activity decays with an estimated half-life of 5 min. The residual activity thereafter decays relatively slowly with a half-life of about 90 min (Sharma, 1976). As the temperature is raised, besides an increase in the initial rate of inactivation, the tail regions of the curves also become progressively steeper. It is apparent that heat-inactivation of adenylate cyclase under our experimental conditions does not follow first-order kinetics. As a first approximation, within limits of experimental error, the decay curves can be described as a sum of two negative exponentials. The half-lives of the fast-decaying component have been estimated to be 5, 3·4, 1·2 and 0·5 min at 37, 40, 44 and 48°C, respectively. The corresponding values for the slow-decaying component- 90, 30, 11 and 5 min are about ten-fold higher.

Figure 1B describes the decay curves for PGE$_1$-stimulated activity. Duplicate samples taken in the experiment of figure 1A were assayed for adenylate cyclase activity in the presence of 10^{-5} M PGE$_1$. These curves, though qualitatively resembling those in figure 1A, show significant quantitative differences as manifested in a downward shift. This is clearly brought out in figure 2 which summarises results of another experiment.



It may be pointed out that a higher initial inactivation rate for PGE$_1$-stimulate activity as compared to that of basal activity (figure 2) has been consistently observed a all temperatures, and can be readily verified by superimposition of the pairs of curve shown in figure 1.

As also seen in figure 2, in contrast to PGE$_1$-stimulated enzyme activity, the initia inactivation rate for etorphine-inhibited activity is significantly lower than that of basa activity. This results in an upward shift of the etorphine-inhibited curve with referenc to that of basal activity. This contrasting behaviour has also been observed for othe stimulatory (fluoride) and inhibitory (carbamylcholine) effectors of adenylate cyclase

Figure 3A summarises results of another experiment in which the loss of etorphine inhibited adenylate cyclase activity is estimated following varying periods of inactiv ation at 45°C. In this case enzyme activities are plotted against time withou normalization with respect to '0' time level in order to bring out certain additiona features of the inactivation process, which are not immediately apparent from figure

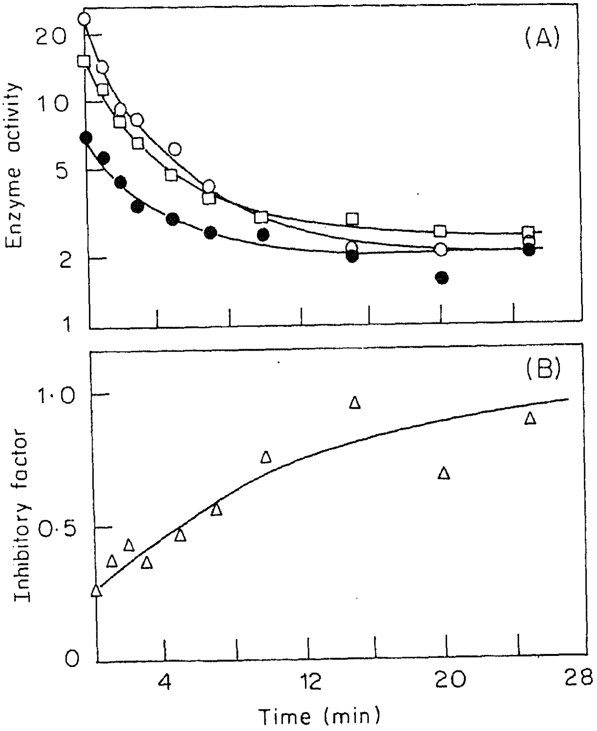


Figure 3. In these studies enzyme preparation (cell homogenate) was incubated at 45°C varying lengths of time. Enzyme activities in the samples were assayed in the presence etorphine (10^{-6} M), etorphine (10^{-6} M) + naloxone (1 mM) and in the absence of effec

Note that the inhibitory response of adenylate cyclase to etorphine, which is maximum at '0' time, is gradually abolished, and the levels of enzyme activity both in the presence and absence of etorphine approach the same value following prolonged inactivation. Naxolone reversed the inhibition of enzyme activity due to etorphine.

This is clearly indicated in figure 3B in which the inhibitory factor, defined as the ratio of adenylate cyclase activity in the presence of etorphine to the basal activity, is plotted against duration of exposure to 45°C. The ratio tends to approach 1 following prolonged inactivation. In contrast, the responsiveness of the enzyme to PGE_1 declines slightly only during the early period of inactivation, and remains constant thereafter. We further note that in the presence of naloxone, a potent antagonist of etorphine, the observed upward shift of the decay curve is greatly reduced. This is important, as it adds to our contention that these displacements are not due to spurious factors, and are related to specific effects of the modulator molecules.

The experiments described above have been carried out with the enzyme present in whole homogenate of the cells made in *tris*-sucrose buffer. In choosing such a system, admittedly a crude one, we were guided by the conviction that a minimal amount of structural disorganisation is an essential pre-requisite for an understanding of the 'native' state of the enzyme, particularly with reference to various endogenous modulators. It is conceivable that such a preparation may contain unbroken cells, or a fraction of the enzyme molecules may have been released from their native environment, *i.e.*, membrane. Heterogeneity introduced thereby may give rise to complex decay curves.

In the experiments presented in figure 4, intact cells and partially purified particulate preparations containing most of the enzyme activity were exposed to 45°C for varying periods of time. There are two points that need to be emphasized here: (i) the decay curves for both intact cells and membrane preparation are multi-exponential, and qualitatively resemble those obtained for the enzyme in whole homogenates and (ii) heat-sensitivities of the enzyme as inferred from initial inactivation rates are markedly different in the three cases.

Since the cells used in these studies have been derived from a hybrid cell line, the possibility that the observed multi-exponential decay merely reflects the presence of two distinct molecular species of adenylate cyclase corresponding to parental cell genomes, cannot be excluded *a priori*. To answer this question we investigated the heat-sensitivity of particulate enzyme preparation from the parental glioma cells and from one day old chick brain. As seen in figure 5, the inactivation kinetics of the enzyme from glioma cells is qualitatively similar to that from neuroblastoma × glioma hybrid cells. The enzyme from glioma cells, however, is significantly more stable than that from hybrid cells. Adenylate cyclase from chick brain shows still more pronounced resistance to heat-inactivation. The physical significance of these observations, particularly with reference to an altered heat-sensitivity of adenylate cyclase from parental glioma cells is not clear at present.

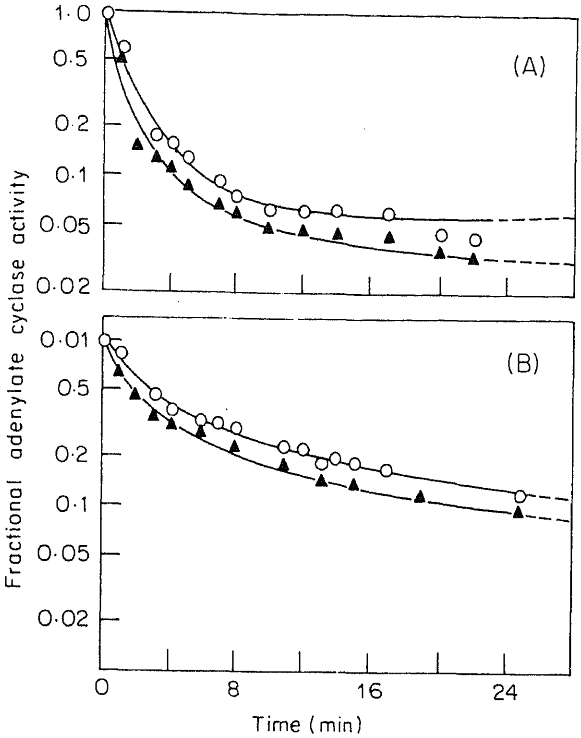


Figure 4. Heat-sensitivity of adenylate cyclase in the particulate (membrane) preparation and the intact cells. In this experiment membrane fraction (A) and intact cells (B) were incubated at 45°C for varying periods and the enzyme activities were assayed in the absence (O) and presence (▲) of etorphine as described in text.

and Weiss, 1977). This apparently simple behaviour is probably due to the fact that the number of possible conformational states of a protein molecule possessing biological activity is severely limited. Parametric analysis of inactivation process as a first-order kinetics in such situations is relatively straightforward, and perfectly justifiable, at least at an operational level. Perhaps, we should mention that often a deeper insight into the inactivation process has been gained from the studies of enzymes that show profound deviation from first-order kinetics (Joly, 1965; Johnson *et al.*, 1954). In recent years, however, most workers, primarily interested in an empirical analysis of alterations in heat-sensitivity of enzymes brought about by binding of specific ligands, either limit their analysis to initial rates, or consider only the early parts of the decay curves to define pseudo-first-order rate constants. Such practices, arbitrary as they are, have

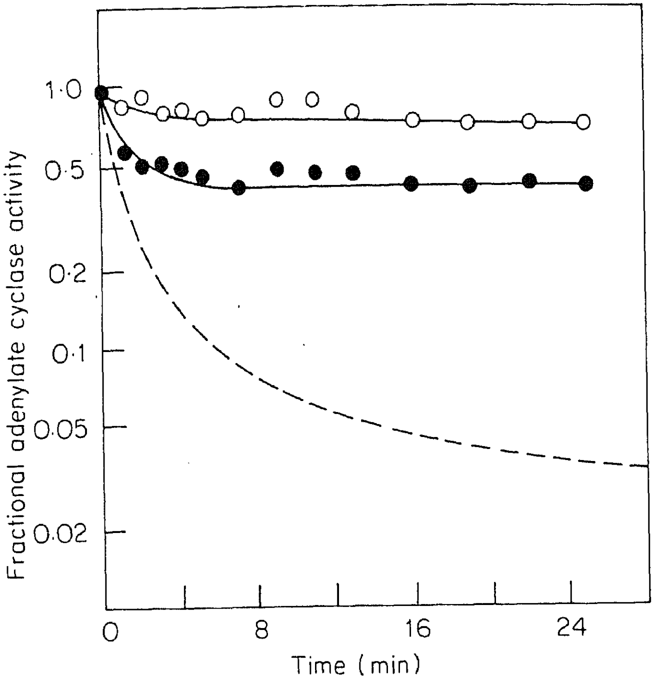


Figure 5. Temperature-inactivation of adenylate cyclase from glioma cells (●), and chick brain (O). Homogenate preparation from the two sources were incubated at 45°C for varying lengths of time. Basal enzyme activities were assayed as described in the text. The dotted curve included in the figure indicates higher heat-sensitivity of adenylate cyclase in N × G hybrid cell homogenates under identical experimental conditions.

biological activity. However, it is also borne out from experience that under constant environmental conditions the time-course of inactivation is fairly reproducible.

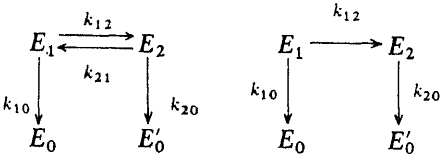
This also holds true in the present case, where we subjected adenylate cyclase from different batches of preparation to inactivation at 45°C under a constant ionic environment (homogenizing medium). Fairly consistent results obtained in all experiments, indeed, prompted us to examine various schemes consistent with observed inactivation kinetics.

In considering inactivation pathways for adenylate cyclase we limit our analysis to schemes that give rise to decay curves comprising two negative exponentials. Consideration of higher order schemes appears unwarranted at the present stage. The general expression in this case is written as:

$$p(E) = (1 - C)e^{-k_r t} + Ce^{-k_s t}, \tag{1}$$

where $p(E)$ represents fraction of enzyme activity surviving at time 't' – $p(E)$ being 1 at o interaction among them. The latter implies that C in eq. (1) should be an invariant, nd have the same value at all temperatures. This is contrary to the data presented in gure 1A for inactivation of basal adenylate cyclase activity in which the values of itercept (C) have been estimated to be 0·60, 0·38, 0·26 and 0·13 at 37, 40, 44 and 48°C, espectively. This large and systematic variation in C, we believe, cannot be wholly ttributed to error inherent in visual extrapolation.

Two minimal inactivation pathways for adenylate cyclase that are compatible with q. (1) are outlined below.



(A) (B)

$_1$ in both the schemes (A and B) represent adenylate cyclase in its 'native' state. E_0 and $'_0$ define irreversibly denatured (inactive) states of the enzyme. An essential difference etween the two mechanisms is that while in A, E_2 represents a reversibly denatured inactive) state, in B, it is envisaged as a distinct conformational state of the enzyme with artial or no loss of biological activity.

It may be pointed out that scheme A and its higher order variations have been xtensively used to describe multi-exponential decay of enzymes (Joly, 1965; Johnson *et l.*, 1954). Perhaps, we should mention that an unambiguous demonstration of eversible step in A is not always straightforward, presumably due to excessive rreversible inactivation as well as due to general lowering of the reaction rate at lower emperatures. Our failure to obtain conclusive evidence in support of reversible nactivation step for adenylate cyclase, indeed, prompted us to consider the alternative cheme B. However, we wish to emphasize that this has not been envisaged on purely rbitrary considerations. It is conceivable that adenylate cyclase in its 'native' state in he particulate fraction may undergo irreversible transition due to 'melting' of nembranous structure at an elevated temperature. While this may involve only partial r no loss of enzyme activity, the released enzyme molecules are likely to have an altered eat-sensitivity.

Analytical solutions of the differential equations for schemes A and B can be readily btained in the form described by eq. (1). Without getting into algebraic details we ummarise the relationships between various rate constants and experimentally derived arameters C, k_r and k_s. For A we have:

$$C = \frac{1}{1+k_e},$$

$$k_r = k_{12} + k_{21},$$

$$k_{10} + k_{20}k_e \qquad (2)$$

by:

$$C = \frac{k_{12}}{k_{10}+k_{12}-k_{20}},$$

$$K_r = k_{10}+k_{12}, \tag{3}$$

$$k_s = k_{20}.$$

C in eqs (2) and (3) can be readily obtained from the intercept on the ordinate by linear extrapolation of the slow-decaying component in a semi-logarithmic plot as shown in figure 1. k_r and k_s represent experimentally derived exponential rate constants from the curves presented in figure 1. From these experimentally determined parameters (C, k_r and k_s) relevant rate constants (k_{12}, k_{21}, k_{10}, k_{20} etc.) can be evaluated for the two schemes shown in A and B.

Differential heat-sensitivity of PGE$_1$- and etorphine-mediated adenylate cyclase activity

Systematic and consistent displacements of the decay curves for stimulatory (figures 1 and 2) and inhibitory (figure 2) response of adenylate cyclase in reference to that for basal activity bear strong relevance to the differential heat-sensitivity of effector-modulated enzyme activity. The existence of heat-labile components or factors as being responsible for modulation of enzyme activity by effector molecules offers the most plausible explanation for the observed shifts. As a general rule, the decay curves representing loss of enzyme activity assayed in the presence of a stimulatory ligand mediated by a heat-labile component will be shifted downwards—the reverse being true in the case of inhibitory molecules. We must reiterate that this contention is valid irrespective of the nature of inactivation mechanism one invokes in order to account for the observed behaviour of basal enzyme activity.

As regards to the functional organization of adenylate cyclase-receptor complex there are two possibilities: (i) the enzyme and receptor protein exist in dynamic equilibrium or (ii) as a preformed complex stabilized in membrane by endogenous factors. We have made an attempt to obtain a rough estimate of the heat-sensitivity of the labile component from the observed displacements of the decay curves for the stimulatory and inhibitory response of the enzyme to PGE$_1$ and etorphine, respectively, in accordance with (ii). We assume that the integrity of both the catalytic (adenylate cyclase activity) and regulatory (receptor protein) sites is essential for the enzyme to elicit stimulatory or inhibitory response. If E is a set of all active molecules, and R that of putative receptors (regulatory sites), then from elementary set theory we can write:

$$E = (E \cap R) \cup (E \cap \overline{R}). \tag{4}$$

Since R and $\overline{R}$ constitute mutually exclusive events, rewriting eq. (4) in terms of probability measure we have:

$$S_t \times p(E) = S_t \times p(E) \times (p(R) + p(E) \times (1 - p(R))), \tag{5}$$

$$p(R) = \frac{S_t - 1}{S_0 - 1}, \tag{6}$$

vhere S_0 refers to the stimulatory factor at time '0'. A similar expression for the decay of eceptor-mediated inhibitory response can be written as:

$$p(R) = \frac{1 - I_t}{1 - I_0}. \tag{7}$$

$_t$ and I_0 now refer to the inhibitory factor at time 't' and '0', respectively.

S_t and S_0, as well as I_t and I_0, can be readily estimated from the data presented in igure 2. Figure 6 shows decay curves for putative receptors of PGE$_1$ and etorphine alculated on the basis of eqs (6) and (7).

It may be pointed out that Burton *et al.* (1976) had earlier reported multiexponential nactivation kinetics for adenylate cyclase at 37°C. However, these observations have emained little more than phenomenological description and no plausible scheme has een suggested. Viewed in this context the analysis presented here should be regarded

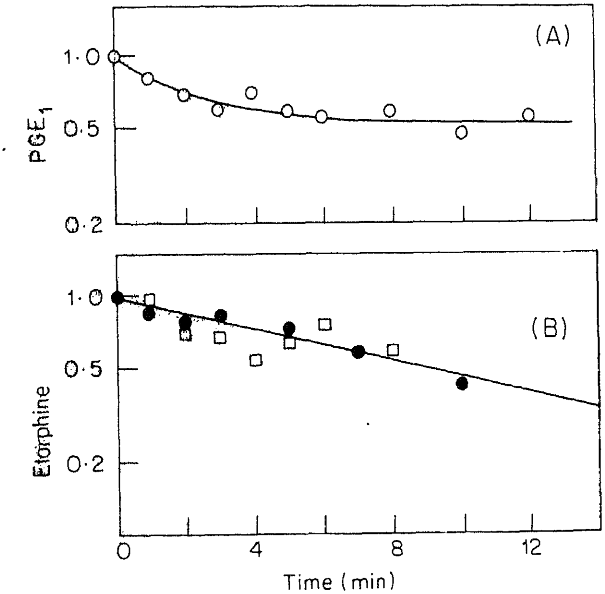


Figure 6. Temperature-inactivation curves for the labile components (receptors) responsible for the stimulatory and inhibitory responses of adenylate cyclase to PGE$_1$ and etorphine, respectively. Data presented in figure 2 were used to calculate $p(R)$ for PGE$_1$ and etorphine in accordance with eqs (6) and (7). It is noticed that while the decay curve for PGE$_1$ receptor (A) is a complex one, that for etorphine receptor (B) is approximately linear over a period of 12 min with an estimated half-life of 8 min.

as an attempt towards the development of a formal model, which may provide a basis for quantitative evaluation of the data.

It is of interest to note that Burton *et al.* (1976) also observed a downward shift in the curves for PGE_1 and fluoride stimulated adenylate cyclase activity as compared to those for basal activity. The model proposed here offers a plausible explanation for these observations, and is based on the contention that there exist two independent sites—catalytic and regulatory—and that the integrity of both these sites is essential for the responsiveness of the enzyme to stimulatory (PGE_1) and inhibitory (etorphine) ligand. As regards the inactivation of PGE_1-binding activity (Burton *et al.*, 1976), it is important to bear in mind that physical binding of the effector molecule to receptor defines a condition that is necessary but not sufficient to elicit stimulatory or inhibitory response. The fact that the inactivation of PGE_1-binding activity is relatively slow as compared to that of PGE_1-stimulated adenylate cyclase activity merely indicates that integrity of catalytic site is not essential for the physical binding of the effector molecules. The predicted inactivation curves for PGE_1 receptors (figure 6), on the other hand refers strictly to their functional integrity, *i.e.*, to the enzyme molecules in which the catalytic sites are conserved.

A relevant question which admittedly cannot be resolved by kinetic studies is – whether the complex inactivation curve for adenylate cyclase reflects different conformational states of the enzyme molecules *per se*, or it is a reflection of the coupling of the receptor to the nucleotide regulatory protein and the catalytic unit in the fluid membrane.

It is interesting to note that heat-inactivation of a related enzyme – cyclic nucleotide phosphodiesterase from normal and leukemic lymphocytes follows a simple first order kinetics over a period of 2 h, with estimated half-lives of 45 and 25 min, respectively (Hait and Weiss, 1977).

The studies presented here have enabled us to arrive at some definitive conclusions which may be summarised as follows:

(i) The observed decay kinetics of adenylate cyclase is not due to two distinct molecular species of the enzyme molecule with little or no interactions among them. This is clearly borne out by a systematic variation in the intercepts on the ordinate by an extrapolation of the slow decaying component at different temperatures of inactivation.

(ii) The kinetic data are shown to be consistent with a model based on the existence of two conformational states of the enzyme molecules.

(iii) Subtle shifts in the inactivation curves for the stimulatory (PGE_1) and inhibitory (etorphine) responses of adenylate cyclase are highly suggestive of differential heat-sensitivities of the corresponding regulatory (or receptor) sites. We wish to emphasise particularly the contrasting behaviour with an upward shift for PGE_1 against a downward shift for etorphine.

(iv) A formalism is developed which permits a quantitative evaluation of the thermal

cknowledgements

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Developmental changes of the glycolytic enzymes in the human fetal heart

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Abstract. The ontogeny of hexokinase, phosphofructokinase, phosphoglucoisomerase, aldolase, pyruvate kinase and lactate dehydrogenase activities which are associated with glycolysis, an important energy yielding process, has been studied in human fetal heart for periods ranging from 13 weeks to above 33 weeks of gestation. Hexokinase, phosphoglucoisomerase and pyruvate kinase activities show similar developmental profiles exhibiting maximum activity at 25–28 weeks of gestation. Phosphofructokinase activity, on the other hand, shows a minimum at this period and exhibits a peak value at early stages (13–16 weeks of gestation). Though considerable activity for aldolase is observed at an early period, it declines thereafter, but again increases in the later period. The probable role and correlations of these glycolytic enzymes with energy demand and general functional development in human fetal heart in ontogeny are evaluated.

Keywords. Glycolysis; hexokinase; phosphoglucoisomerase; phosphofructokinase; aldolase; pyruvate kinase; lactate dehydrogenase; human fetal heart; development.

ntroduction

n the past few years, a great deal of interest has been centred upon carbohydrate metabolism, particularly on the glycolytic pathway during development in various pecies. Similar to other organs, the relative importance of the glycolytic pathway, in comparison with other pathways, has been demonstrated in normal heart slices (Charles nd Sidney, 1956). Like adult brain, adult cardiac muscle also shows a great dependence n this pathway for deriving energy (Beatty *et al.*, 1972). Hahn and Skala (1971) and Cox and Gunberg (1972) have stressed the importance of this glycolytic pathway in arly life of rat fetal heart also. Similarly, Wittels and Bressler (1965) have demonstrated that energy required for contraction of bovine fetal heart is mainly supplied by arbohydrate metabolism.

Despite all the impressive evidence regarding the existence and function of the lycolytic pathway in developing mammalian heart, little attention has been paid to a study on human fetal heart. It has been pointed out that in adult human heart, the lycolytic pathway is active (Jolley *et al.*, 1958). Therefore, the present study was

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biochemical parameters to the structural and functional ontogeny of human fetal heart and may also provide a gauge for the developmental process and the modifications in the enzymes of the glycolytic pathway. Six important glycolytic enzymes namely, hexokinase, phosphoglucoisomerase, phosphofructokinase, aldolase, pyruvate kinase and lactate dehydrogenase have been selected for this study and their activities have been followed in the isolated human fetal heart during different gestational period.

Materials and methods

The human fetuses of different gestational ages were obtained as therapeutic abortions from different MTP (Medical termination of pregnancy) clinics and hospitals in and around Calcutta. Fetuses above 20 weeks were collected as stillborn. Beginning from 13 weeks, the groups were divided at 3 week intervals up to 32 weeks and one group included a period of 33 weeks and above (also see Das *et al.*, 1979). Six samples were experimented in each group. Immediately after the heart tissue was removed it was left in the deep freeze (–20°C) for 7–8 h. The tissue was then homogenised with deionized water (10%, w/v) and after centrifugation at 1,000 *g* for 15 min at 0–5°C the supernatant fraction was used as the source of various enzyme activities studied.

Hexokinase was determined by the coupled enzyme assay method of Joshi and Jagannathan (1966) with glucose and adenosine triphosphate (ATP) as substrates and glucose-6-phosphate dehydrogenase as the second enzyme. Phosphoglucoisomerase was assayed by the method of Roe *et al.* (1949) and pyruvate kinase was assayed by the method of Weber *et al.* (1965). Phosphofructokinase was determined according to the coupled enzyme assay method of Ling *et al.* (1966) with fructose-6-phosphate and ATP as substrates and aldolase as the second enzyme.

Aldolase was determined by the colorimetric method of Beck (1955) and lactate dehydrogenase was assayed by the method of Neilands (1955) with lactate as substrate and nicotinamide adenosine (NAD) as the coenzyme. The data in the figure 1 have been expressed in international units (milliunits/mg protein). Protein content of the samples was determined by the method of Lowry *et al.* (1951).

Results

The developmental patterns of hexokinase, phosphoglucoisomerase and pyruvate kinase activities in human fetal heart are illustrated in figure 1A. It is apparent from this figure that both hexokinase and phosphoglucoisomerase exhibit a similar time sequence during development. Both the enzymes have a high level of activity at early stages of development (*i.e.* 13–16 weeks of gestation) and drop sharply during 17–24 weeks of gestation. The maximum activity for both the enzymes is observed at 25–28 weeks of gestation, after which the activities fall to lower levels and at 33 weeks and above it represents about 38% of the maximum activity in both cases. The activity-

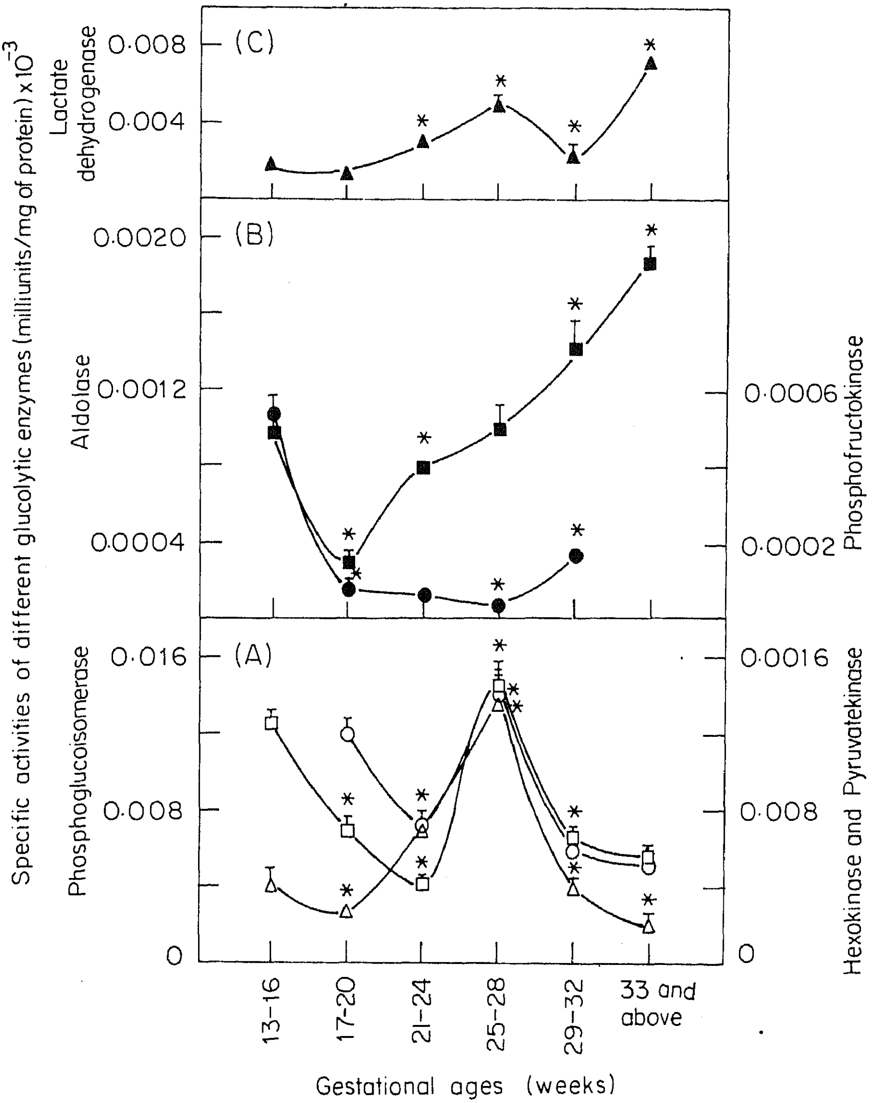

Figure 1. **A.** Developmental changes of hexokinase (O), pyruvate kinase (Δ) and phosphoglucoisomerase (□) in human fetal heart. **B.** Developmental changes of aldolase (■) and phosphofructokinase (●) in human fetal heart. **C.** Developmental changes of lactate dehydrogenase (▲) in human fetal heart.

Values represent mean and vertical bars represent S.D. ($n = 6$ in each group). * $P < 0{\cdot}001$ (highly significant).

hoglucoisomerase, but in contrast to these enzymes, the rise in activity of pyruvate nase begins earlier (after 20 weeks of gestation).

phofructokinase activity is found to be maximum even during 13–16 weeks of gestation and thereafter declines sharply at 17–20 weeks and remains at this low level till 25–28 weeks. After this stage, it shows a tendency to increase. The developmental changes in aldolase activity follow a pattern similar to phosphofructokinase up to 17–20 weeks showing a minimum at this stage, but its pattern abruptly changes thereafter showing a sharp increase even at gestation periods above 33 weeks.

Figure 1C shows the developmental time course of lactate dehydrogenase activity in human fetal heart. Like pyruvate kinase, lactate dehydrogenase starts with a low activity and after 17–20 weeks of gestation rises markedly up to 25–28 weeks of gestation. It drops to a low level at 29–32 weeks after which it shows a second increase till 33 weeks and above.

Discussion

The results presented herein provide a clear picture regarding the developmental profile of enzymes, associated with glycolysis in human fetal heart. Except for aldolase and phosphofructokinase, the other enzymes studied show a peak at 25–28 weeks of gestation. It has been reported in pig heart that both the metabolites glucose-6-phosphate and fructose-6-phosphate inhibit phosphofructokinase activity (Werner *et al.*, 1983). The maximum activity of hexokinase and phosphoglucoisomerase at 25–28 weeks of gestation indicating a greater production of glucose-6-phosphate and fructose-6-phosphate at this stage and the minimum activity of phosphofructokinase at 25–28 weeks of gestation are in good agreement with the above evidence. Besides, it has been well established that the inhibition of phosphofructokinase leads to the accumulation of glucose-6-phosphate which in turn inhibits the activity of hexokinase (Weil-Malherbe and Bone, 1951). The inverse relationship between hexokinase activity and phosphofructokinase activity lends support to the findings cited above.

Neely and Morgan (1974) showed that a decrease in fructose-6-phosphate results in an increase in fructose-1,6-diphosphate associated with faster glycolytic flow. The developmental behaviour of aldolase follows more or less a similar pattern with phosphofructokinase (figure 1B) except for a sudden increase after 17–20 weeks of gestation, the peak being observed at later stages of gestation in fetal heart.

Besides the divergent behavioural profile of lactate dehydrogenase at different stages of gestation may be explained from the turnover characteristics of this enzyme and its multiple form in fetal tissue.

In conclusion it can be said that all the enzymes studied here except phosphofructokinase show very high activities at 25–28 weeks of gestation which may correspond to the appearance of a rapid movement of human fetus and high fetal heart rate during this period indicating a rapid development of human fetal heart (Sorokin *et al.*, 1982).

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drodynamic properties of α-globulin from *Sesamum indicum* L.

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Abstract. The protein α-globulin from *Sesamum indicum* L. has been characterised for its size and shape using various chemical, physico-chemical and hydrodynamic properties. The protein has an $S^0_{20,w}$ of 12·8, $D_{20,w}$ of $4{\cdot}9 \times 10^{-7}$ cm²/sec and a partial specific volume of 0·725 ml/g in the native state. The intrinsic viscosity of the protein was determined to be 3·0 ml/g indicating it to be globular in shape. The molecular weight of the protein as determined by various approaches in analytical ultracentrifugation varies from $2{\cdot}6$–$2{\cdot}74 \times 10^5$. The molecular weight from sedimentation equilibrium yields a value of $2{\cdot}74 \times 10^5$ in the native state and a value of 19000 in the dissociated and denatured state in 6 M guanidine hydrochloride. The evaluation of frictional ratios using Stokes radius and results from electron microscopy confirms the protein to be globular in shape. The protein consists of at least 12–14 subunits. The evaluation of hydrophobic parameters and energetics of interaction of subunits indicate that the protein is stabilized predominantly by hydrophobic interactions.

Keywords. α-Globulin; subunits; sesame seed; hydrophobic.

roduction

ysicochemical characterization of seed proteins is important in order to understand nature of the protein, its interaction with other cell components and more portantly the energetics of the stability of the molecule. The major protein of *amum indicum*, α-globulin, constitutes ~ 65–70 % of the total proteins present in the d (Prakash and Nandi, 1978). The protein has been isolated and characterized (Nath *al.*, 1957; Sinha and Sen, 1962; Ventura and Lima, 1963; Prakash, 1976). The ociation–dissociation and denaturation behaviour of α-globulin under various ution conditions of electrolytes, detergents, urea, guanidine hydrochloride (GuHCl), d and alkali are studied in detail (Prakash and Nandi, 1976a, b, 1977a, b, c, 1978, 32; Prakash, 1980, 1982; Prakash *et al.*, 1980; Lakshmi and Nandi, 1977, 1978). cently it was shown that α-globulin is rich in aperiodic and β-structure (Prakash, 30; Prakash *et al.*, 1980; Prakash and Nandi, 1982).

n the present study in order to determine the probable size and shape of α-globulin, protein isolated by a nondenaturing procedure has been studied from a drodynamic angle and several physical parameters such as molecular weight, fusion co-efficient, Stokes radius, partial specific volume, intrinsic viscosity,

have been used to understand the various forces which hold the subunits of α-globu
as a thermodynamically stable protein unit.

Materials and methods

Sesame seeds of authentic variety were obtained commercially and α-globulin w
isolated from the defatted flour (Prakash and Nandi, 1978).

The chemicals and reagents used were as follows and the source of these material
shown in the parenthesis: Sepharose 6B-100, 2-mercapto ethanol, GuHCl (Sig
Chemicals); sodium chloride and potassium hydrogen phosphate (BDH Chemica

α-Globulin was isolated in a homogeneous form by the previously described meth
(Prakash and Nandi, 1978). Solutions of α-globulin in 0·1 M phosphate buffer pH
containing 1 M NaCl (PS buffer) were prepared and used in all the experiments unl
otherwise stated.

Determination of protein concentration

Protein concentrations were measured routinely by ultraviolet absorption using a val
of 9·54 dl/g-cm as the extinction coefficient of α-globulin in PS buffer (Prakash, 198

Velocity sedimentation

Velocity sedimentation experiments were carried out in PS buffer and also in 6
GuHCl in a spinco Model E analytical ultracentrifuge. A standard 12 mm single sect
Kel F Centerpiece was used, and the experiments were carried out at ~ 25°C unl
otherwise stated and at 59,780 rpm. Plates were read in a Gaertner microcomporat
and $S_{20,w}$ values calculated by standard procedure (Schachman, 1957).

Diffusion experiments

Diffusion experiments were conducted in Spinco Model E analytical ultracentrifu
using a synthetic boundary cell at a rotor speed of 8600 rpm with a prote
concentration of 2–12 mg/ml at 25 ± 1°C. Diffusion coefficient was calculated by t
height area method described by Kawahara (1969).

Viscosity

Viscosity measurements were made using an Ostwald Viscometer in the presence
6 M GuHCl at temperature of 30 ± 0·1°C. The intrinsic viscosity determined fro
extrapolation of reduced viscosities determined at different protein concentrations
zero protein concentration (Yang, 1961; Bradbury, 1970).

Molecular weight determination

Molecular weight of α-globulin was determined from a combination of sedimentati
coefficient value and intrinsic viscosity both in buffer and in the presence of 6 M GuH

iolecular weight by Archibald approach to equilibrium and molecular weight by edimentation and diffusion methods were determined as described by Schachman 1959). The molecular weight by sedimentation equilibrium was determined according o the procedure of Yphantis (1964). Point average molecular weight was calculated oth for the native as well as denatured protein in 6 M GuHCl.

tokes radius

tokes radius of the protein was determined by gel chromatography in Sepharose 6B-00 column employing 0·04 M sodium phosphate buffer of pH 8·0 containing 5 mM DTA. The column was equilibrated with the above buffer and calibrated with the tandard proteins, urease, bovine serum albumin, ovalbumin, α-chymotrypsin and ibonuclease. The Stokes radius R_s was evaluated by the procedure of Ackers (1967).

rictional coefficients

he frictional coefficient of α-globulin was calculated by several approaches as reviewed y Siegel and Monty (1966).

Iydrophobicity

he average hydrophobicity and related terms were calculated according to the rocedure of Bigelow (1967).

lectron microscopy

he protein samples at low concentration was treated with 0·5% glutaraldehyde, dsorbed to carbon coated formavar grids, stained with 1% uranyl acetate and bserved in Philips EM 3001, electron microscope.

esults and discussion

n most of the hydrodynamic studies it is preferable to perform the experiments near he isoelectric point of the protein to reduce the charge effects. Since the solubility f α-globulin was very poor both in buffers of low ionic strength and in buffers of high onic strength in the region of isoelectric pH (pH 4–5), studies were carried out at pH 6·5 n 0·1 M phosphate buffer containing 1 M NaCl unless otherwise stated *i.e.*, PS buffer. lso the parameter concerned was looked into as a function of protein concentration nd the results are tabulated in table 1.

Hydrophobicity and related parameters

The average hydrophobicity and related parameters like frequency of nonpolar side hains (NPS) *i.e.*, nonpolar residue fraction and polar ratio (P) were calculated and are

Table 1. Hydrodynamic parameters of α-globulin.

Parameters	Values
1. Hydrophobicity and related values[a]	
(a) Average hydrophobicity	872 cals/residue
(b) NPS	0·26
(c) P	1·36
(d) Charge	0·362 units/residue
2. Sedimentation coefficient ($S^0_{20,w}$)[b]	
(a) Native	12·8 S ± 0·1 S
(b) 6 M GuHCl ($S_{20,w}$)[c]	2·0 S ± 0·2 S
3. Diffusion coefficient ($D_{20,w}$)[d]	$4{\cdot}9 \times 10^{-7}$ cm²/sec
4. Intrinsic viscosity [η][e]	
(a) Native	3·0 ± 0·2 ml/gm
(b) 6 M GuHCl	38·5 ± 1·0 ml/gm
5. Non ideality coefficient (g)[a]	0·01 ml/mgm
6. Partial specific volume[f]	
(a) Native	0·725 ± 0·002 ml/gm
(b) 6 M GuHCl	0·684 ± 0·002 ml/gm
7. Hydrated volume (Ve)[g]	0·711 ml/gm
8. Hydration factor (φ)[h]	0·27 gm of water/gm of protein
9. Molecular weight	
(a) Approach to sedimentation equilibrium[i]	2,50,000 ± 20,000
(b) Sedimentation and diffusion measurement[j]	2,36,000 ± 15,000
(c) Sedimentation and intrinsic viscosity[k]	2,52,000 ± 15,000
(d) Sedimentation equilibrium[l]	
(i) Native Mn (number average)	2,65,000 ± 12,000
Mw (weight average)	2,74,000 ± 14,000
(ii) 6 M GuHCl	
Mw (number average)	18,000 ± 1,000
Mw (weight average)	19,000 ± 1,500
10. Size	
(a) Stokes radius[m]	37 ± 3 Å
(b) From sedimentation measurements[n,o]	47 ± 4 Å
(c) From diffusion measurements[n,o]	43 ± 4 Å
(d) Radius of gyration[p]	29 ± 3 Å
11. Frictional ratios[n,o]	
(a) From radius of equivalent sphere	1·00 ± 0·05
(b) From sedimentation and molecular weight	1·10 ± 0·1
(c) From Stokes radius and hydrated volume	1·00 ± 0·15
12. Shape parameters[q,r]	
(a) β-function	$2{\cdot}18 \times 10^6$
(b) Perrin shape factor	1·50 ± 0·1
(c) Simha shape factor	4·17 ± 0·5
13. Axial ratios[q,r]	
(a) Prolate ellipsoid of revolution	3·5

Table 1. (*Contd.*)

Parameters	Values
14. Subunit number	
(a) SDS-PAGE[i]	12 (6 × 2) (minimum)
(b) 6 M GuHCl[l]	14 (7 × 2)
15. Subunit interactions	Predominantly hydrophobic

[a]Bigelow (1967); [b,j,k]Schachman (1957); [c]Prakash and Nandi (1977a); [d]Kawahara (1969); [e]Bradbury (1970); [f]Prakash (1982); [g,h]Haschemeyer and Haschemeyer (1973); [i]Prakash and Nandi (1977b; 1978); [l]Yphantis (1964); [m]Ackers (1967); [n]Tanford *et al.*, (1974); [o]Siegel and Monty (1966); [p]Pilz (1973); [q]Simha (1940); [r]Scheraga and Mandelkern (1953).

rotein of safflower seed protein (Latha and Prakash, 1986). Carmin has an NPS value f 0·2 and charge of 0·609 units/residue. These results indicate that α-globulin is rich in ydrophobic amino acids and possibly many of its properties are governed by this high ontent of hydrophobic amino acids like, solubility, heat coagulation, subunit–subunit nteractions etc. However one has to be cautious in using these empirical hydrophoicity values with the structure–function relationships.

elocity sedimentation

he sedimentation coefficient determined as a function of protein concentration is hown in figure 1. From the graph $S^0_{20,w}$ value of 12·8 ± 0·1 was obtained for α-globulin. he concentration dependence showed the usual negative slope and was analysed ccording to the standard procedure (Schachman, 1959). The analysis yielded a value or non-ideality coefficient g = 0·01 ml/mgm (Schachman, 1957).

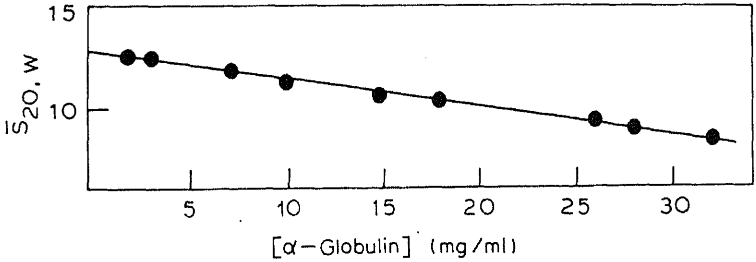

Figure 1. Concentration dependence of the $S_{20,w}$ value of α-globulin in PS buffer.

iscosity

[illegible] function of protein concentration over

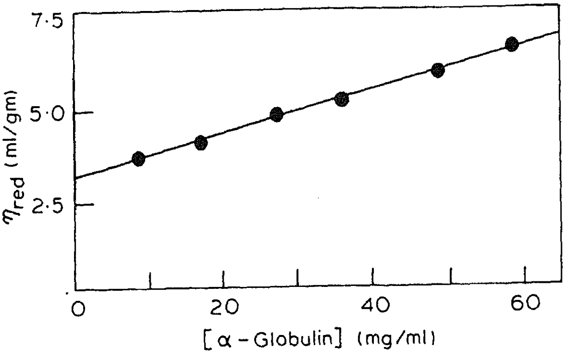

Figure 2. Concentration dependence of η_{red} of α-globulin in PS buffer.

globulin in the native state (Tanford, 1961). Prakash and Nandi (1977) also reported a value of 3·0 ml/gm as the viscosity of α-globulin in 0·02 M phosphate buffer of pH 7·5 containing 1 M NaCl. In figure 3 is shown the plot of reduced viscosity *versus* protein concentration in 6 M GuHCl for which an intrinsic viscosity of 38·5 ml/gm is obtained. This value is in the region for random coils as reported by Tanford (1968).

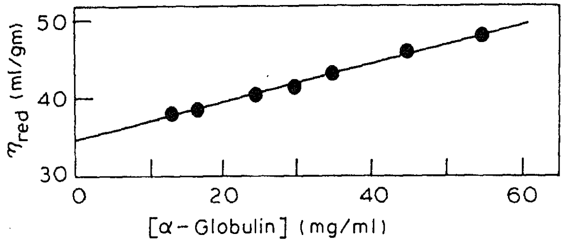

Figure 3. Concentration dependence of η_{red} of α-globulin in 6 M GuHCl containing 0.1 M of 2-mercaptoethanol.

Table 1 lists the molecular weights calculated by various hydrodynamic approaches. The values indicate the protein to be of high molecular weight of around 0·27 million. From the results one can see that a combination of sedimentation and diffusion measurements gives the lowest value of $2{\cdot}36 \times 10^5$ whereas sedimentation equilibrium in native state shows the highest value of $2{\cdot}74 \times 10^5$ (figure 4A), others being in between these two values (table 1). Figure 4A shows a plot of *ln* (f) *versus* r^2 of the

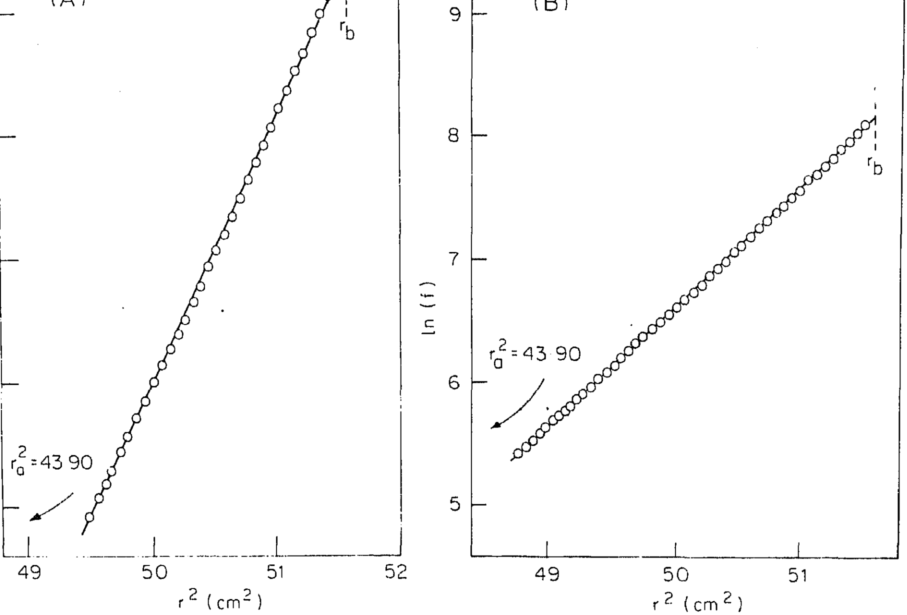

Figure 4. Sedimentation equilibrium patterns of α-globulin in (a) PS buffer and (b) in 6 M GuHCl respectively containing 0.1 M of 2-mercaptoethanol at 20°C. The speed of the run and the concentration of the protein used respectively is indicated against each photograph. The results are plotted as *ln* of fringe displacement *versus* (radial distance)^2. (a) 12,000 rpm; 480 μg/ml; (B) 32,000 rpm; 300 μg/ml.

ecular weight of other oilseed proteins from various sources are also in the same ge (Prakash and Narasinga Rao, 1984).

es radius and frictional ratios

es radius of the protein was determined experimentally from gel filtration and gave lue of 37 Å. It was also calculated from the experimentally determined sedimenon and diffusion values by using the equations described by Tanford *et al.* (1974). values obtained *i.e.*, 47 Å and 43 Å respectively from sedimentation and diffusion higher as compared to the experimentally obtained Stokes radius. The approximate us of gyration was also calculated by using the appropriate equation for a sphere , 1973) and gave a value of 29 Å. $R_{\min}$ was also calculated assuming α-globulin to be nhydrated sphere. This gave a value of 43 Å which is in excellent agreement with the e calculated from diffusion measurements and also not too far from the erimentally determined Stokes radius value.

rictional ratios were evaluated from the sedimentation, partial specific volume, ecular weight, intrinsic viscosity and hydrated volume by using the appropriate ation. The values are shown in table 1. All the values suggest a frictional coefficient confirm the earlier results of intrinsic viscosity. From the Scheraga and Mandelkern (1953) equation the value of β-function was calculated and it yields a value of $2{\cdot}18 \times 10^6$ from which one can envisage on axial ratio for prolate ellipsoid of resolution of 3·5 and for oblate ellipsoid of resolution of 3·0 respectively. Other relevant shape parameters are also given in table 1.

Subunit composition

The subunit composition of the protein as determined using sodium dodecyl sulphate (SDS)-polyacrylamide gel electrophoresis (PAGE) in the presence of a disulfide reducing agent indicates at least 12 subunits, in the molecular weight range of 8000–85000 (Prakash and Nandi, 1978). On this basis, one can tentatively conclude that the oligomer is made up of the least 12 nonidentical subunits. Sedimentation equilibrium data in 6 M GuHCl in the presence of 2-mercaptoethanol (figure 4) indicate a weight average molecular weight from the straight line fit of the ln (f) *versus* r^2 plot, of 19000 and a number average of 18000. If the native molecular weight is taken as $2{\cdot}74 \times 10^5$, a total number of 14 subunits can be envisaged for α-globulin each of molecular weight 19000. These results are in contradiction to the SDS-PAGE results in terms of the range of molecular weights and also the number of subunits. The anomaly in SDS-PAGE may be due to the presence of nearly 1·0 % carbohydrate in the protein as reported by Prakash and Nandi (1978). Also Segrest *et al.* (1971) and Neville (1971), have indicated the anomalous mobility and behaviour of proteins containing carbohydrates in the SDS-PAGE systems. Further, since figure 4B indicates a straight line with no curvature till the bottom of the cell, the system is homogeneous in itself and the subunits are all probably of equimolecular weight. Further the homogeneity of the system is well established since the number and weight average molecular weights are close to each other within experimental limitations. This is not unusual in the quaternary structure of many proteins whose subunits are of equal molecular weight (Haschemeyer and Haschemeyer, 1973).

Nature of subunit interaction

α-Globulin exists as a single component in 0·5 M Tris/HCl buffer of pH 7·0 with sedimentation coefficient of 13 S (Prakash and Nandi, 1977c). Increase in the pH induces dissociation and at pH 9·0, the 13 S component at 27°C dissociates to nearly 40 % to an 8 S component (Prakash and Nandi, 1977c). The effect of variation of temperature at pH 9·0 indicates that increase in temperature favours the associated 13 S component and suggests qualitatively, hydrophobic interactions of the subunits of α-globulin (Prakash and Nandi, 1977c).

To characterise and quantify the thermodynamic parameters and the nature of the association reaction in α-globulin the following procedure was adopted. From the ratio of concentration of 13 S to 8 S the apparent association constant K for the association reaction at three different temperatures were calculated. A plot of log K as a function of reciprocal of absolute temperature is found to be linear as has been represented in

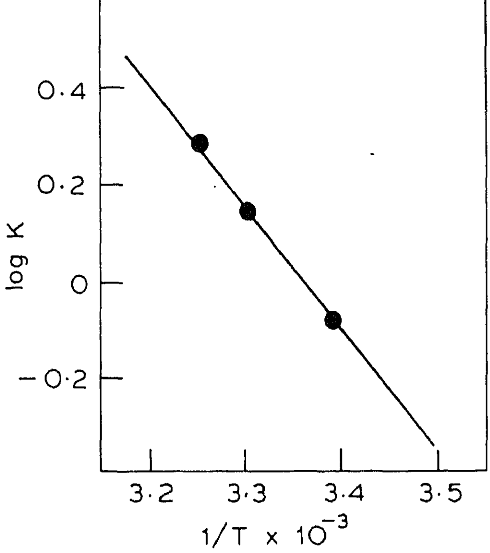

Figure 5. Plot of log K *versus* $1/T$ where K is the apparent association constant of 13 S component and T the absolute temperature.

the association reaction. The free energy

$$\Delta F = -RT \ln K, \tag{1}$$

ere R is the gas constant, T the absolute temperature and K the apparent association istant gives a value of $-0{\cdot}19$ Kcal/mol at 27°C. These results yield a value of ΔS 42 e.u. for the entropy of the association reaction of 27°C using the equation

$$\Delta F = \Delta H - T.\ \Delta S, \tag{2}$$

ere ΔF is the free energy, ΔH is the enthalpy and ΔS is the entropy of the reaction l T is the absolute temperature. This indicates that the association of the subunits of protein α-globulin is predominantly governed by hydrophobic interactions auzmann, 1959).

ctron microscopy

;ure 6 shows the photomicrograph of α-globulin in the electron microscope. A close mination of the photomicrograph indicates cluster or beads or individual beads as own by arrows and indicates the protein to be fairly globular in shape. The absence of ier structure like rods, or ellipsoids confirms the already available data that the otein is globular in shape.

t is important to keep in mind that the dimensions of protein described in this study only approximations assuming either spherical or equivalent ellipsoidal models. ese should be interpreted with caution in describing the real particles.

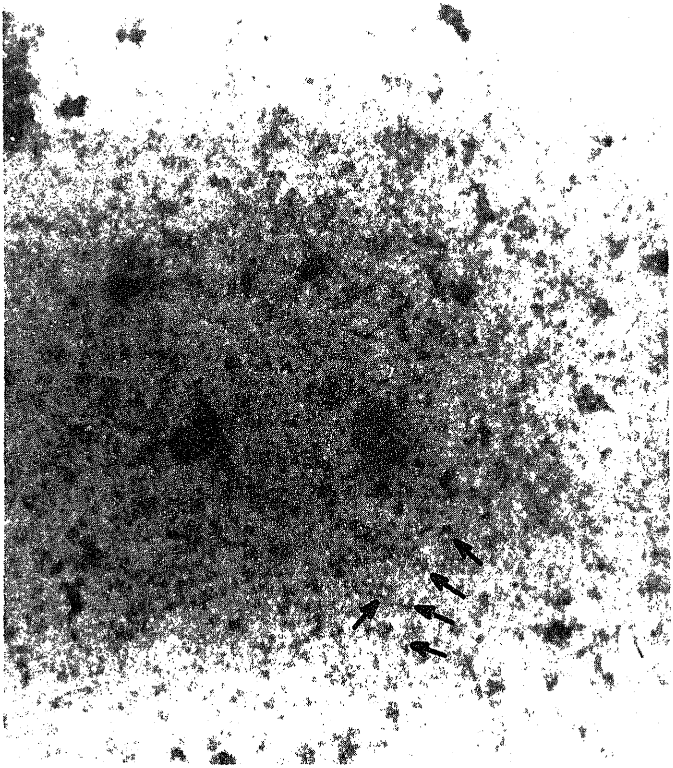

Figure 6. Photomicrograph of α-globulin in the electron microscope at 10000 times magnification factor.

observed $S^0_{20,w}$ is not far off from the range of globular shape for α-globulin. The hydration factor appears to be high (table 1) and this may be due to the high compactness of the protein molecule as reflected by $[\eta]$ of 3·0 ml/gm and as also the high content of hydrophobic amino acids and the clathrate formation around these apolar groups (Jencks, 1969).

The exact interpretation of the overall shape of the protein α-globulin depends very much on the definition of the limiting axial ratio, intrinsic viscosity and the comparison of the various radii along with their respective molecular weights. In conclusion, α-globulin is a highly compacted globular protein of molecular weight 274,000 and is made up of at least 12–14 subunits of equal molecular weights of ~ 19000 predominantly held together by entropically driven hydrophobic interactions with a negative free energy of interaction of 0·19 Kcal/mol.

Acknowledgement

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Association–dissociation of glycinin in urea, guanidine hydrochloride and sodium dodecyl sulphate solutions

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Abstract. The effect of urea, guanidine hydrochloride and sodium dodecyl sulphate on glycinin, the high molecular weight protein fraction from soybean has been investigated by analytical ultracentrifugation. Urea and guanidine hydrochloride dissociate the protein to a '2S' protein through the intermediary 7S and 4S proteins. However, in sodium dodecyl sulphate the protein directly dissociates to a 2S protein. Analysis of the data by calculation of per cent fraction and $S_{20,w}$ value indicates that dissociation and denaturation of glycinin occur simultaneously in the presence of the above reagents but to different extents.

Keywords. Association; dissociation; guanidine hydrochloride; sodium dodecyl sulphate; urea; glycinin; denaturation.

Introduction

Urea and guanidine hydrochloride (GuHCl) and sodium dodecyl sulphate (SDS) are well known protein-dissociating and denaturing reagents and are used extensively in protein chemistry to understand the forces responsible for maintaining the native structure of a protein molecule (Joly, 1965; Tanford, 1968; Jancks, 1969). Both urea and GuHCl are effective at high concentrations (> 6 M) whereas SDS is effective at low concentrations (Tanford, 1968, 1970; Pace, 1975; Reynolds, 1982). Many proteins loose their ordered structure upon addition of these reagents and in multimeric proteins both dissociation and denaturation take place (Tanford, 1968, 1970; Lapanje, 1968; Prakash and Nandi, 1976, 1977; Gururaja Rao and Narasinga Rao, 1979; Rahma and Narasinga Rao, 1981; Prakash and Narasinga Rao, 1984).

The high molecular weight protein component of soybean (*Glycine max*) proteins, termed often as 11S component or glycinin, is a multimeric protein containing 12 non-identical subunits with a molecular weight of 350,000 (Catsimpoolas, 1967). The dissociation of glycinin has been studied in detail mainly with changes in pH, ionic strength, in sodium octyl benzene sulphonate and urea solutions of low concentration (Naismith, 1955; Wolf and Briggs, 1958; Wolf *et al.*, 1958). Wolf and his co-workers have used urea mostly to dissociate the subunits of glycinin. However, urea, GuHCl and quantitation of the number of subunits (Catsimpoolas, 1969; Catsimpoolas *et al*., 1969; Koshiyama and Fukushima, 1976). The present work deals mainly with the dissociation and denaturation of glycinin in urea, GuHCl and SDS solution. The object of this investigation was to determine if there were any differences in the effect of these reagents on glycinin. In addition it was also of interest to determine if dissociation and denaturation of the protein occur simultaneously or sequentially.

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Materials and methods

Materials

Bragg variety soybean (*Glycine max*) seeds cultivated in a farm near Mysore, were purchased locally. Sephacryl S-200, ultrapure urea and GuHCl were purchased from Sigma Chemical Company, St. Louis, Missouri, USA. Stock solutions of concentrated urea and GuHCl were prepared, treated with activated charcoal and then appropriate buffer salts were added to obtain the required pH. The solutions were used within 7 days of preparation (Prakash *et al*., 1981) and β-mercaptoethanol was obtained from Fluka; SDS obtained from Loba Chemie was used after recrystallizing twice with ethanol. Buffer salts, sodium chloride and all other reagents were of analytical reagent grade.

Methods

Isolation of glycinin: Glycinin was isolated by a minor modification of the method of Appu Rao and Narasinga Rao (1977). The protein solution in buffer obtained after final precipitation with ammonium sulphate was purified on Sephacryl S-200 column, the major peak portion pooled and dialysed against large quantities of water for 24 h with repeated changes of water. The solution was then centrifuged at 16000 *g* for 1 h and lyophilized.

Determination of protein concentration: The protein in native state and in different concentrations of denaturant were all taken in 0·05 M sodium phosphate buffer of pH 7·8 containing 0·35 M NaCl and 0·1 % β-mercaptoethanol. For experiments with SDS, NaCl was not added. The protein concentration was determined by absorbance measurements using a value of 7·9 for $E^{1\%}_{1\,\mathrm{cm}}$ at 280 nm (Appu Rao and Narasinga Rao, 1977).

Sedimentation velocity: Sedimentation velocity runs were made with Spinco Model E analytical ultracentrifuge equipped with phase-plate schlieren optics and rotor temperature indicator and control unit. All runs were made using 1 % protein solution prepared with lyophilised protein. The protein solutions were dialysed against the solvent in which they were prepared for 24 h before the runs were made. A standard 12 mm duraluminium or Kel F cell was used for the runs at a speed of 59780 rpm and ~ 27°C. The plates were read on a Gaertner microcomparator or an Abbe Comparator, modified to read ultracentrifuge plates, and $S_{20,w}$ values were calculated by the

Results

Figure 1A, shows the sedimentation velocity pattern of glycinin. It indicates a single major peak with an $S_{20,w}$ value of 11·5 with a small quantity (< 5%) of 7S component

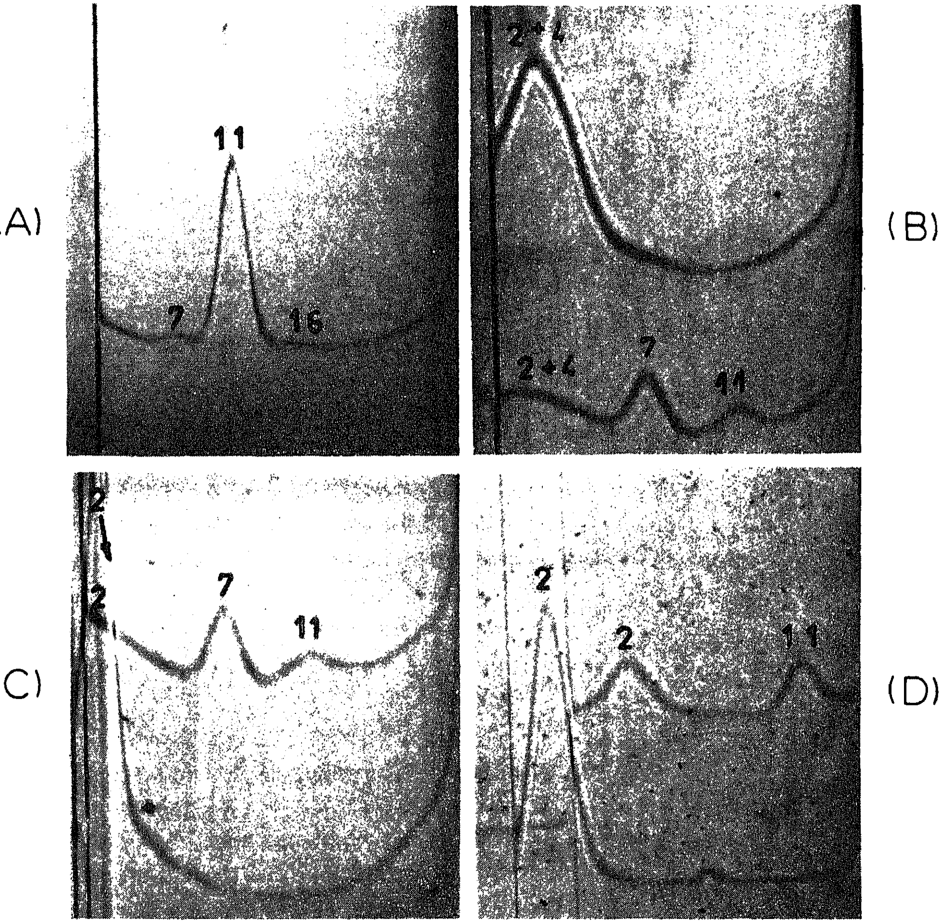

Figure 1. Representative velocity sedimentation patterns of glycinin under various conditions of experimentations. All runs were carried out at 59,780 rpm, 27°C and 1% protein concentration unless otherwise stated. The photographs are taken 32 min after attaining the maximum speed. Glycinin in (A) phosphate buffer, pH 7·8, 0·05 M containing 0·35 M sodium chloride and 0·1% 2-mercaptoethanol; (B) top, 5 M urea; bottom, 2 M urea; (C) top, 1 M GuHCl; bottom, 4 M GuHCl; (D) top, 0·7 mM SDS and bottom, 3·5 mM SDS.

(Wolf *et al.*, 1962, Catsimpoolas *et al.*, 1969). Hence proper corrections are incorporated in analysing the data for the presence of both 7S and 16S components.

Effect of urea

In figure 1B, the velocity sedimentation patterns of glycinin at urea concentrations of 1 M and 3 M are given. The protein dissociates progressively to 7S, 4S and 2S components. However, due to the viscosity of solution, it was difficult to achieve total separation of 2S and 4S components. The following sequence appears to be taking place in the dissociation reaction.

$$11S \rightarrow 7S \rightarrow (4S + 2S) \text{ or LMW component.}$$

The (4S + 2S) component is termed as low molecular weight (LMW) component for the purposes of discussion. From figure 2a it is apparent that even at 1 M concentration, there is considerable LMW component. Also there is a decrease in 11S protein and the 7S protein is also seen. With further increase in urea concentration to 2 M, the 7S protein reaches a maximum concentration, and the LMW component increases rapidly to about 65 %; the 16S protein is absent. At 5 M urea concentration the pattern consists essentially of LMW component. Also the $S_{20,w}$ value of the LMW fraction is 2S and the peak is gaussian in shape indicating the homogeneity of the fraction. Prakash and Nandi (1977) have reported similar observations with the multimeric protein α-globulin of sesame seed where the dissociation is complete at 5 M urea.

Effect of GuHCl

In figure 1C, the velocity sedimentation patterns of glycinin in GuHCl solution of 1 M and 4 M are given.

The protein dissociates in these solutions also (figure 1C). The dissociation follows the sequence

$$11S \rightarrow 7S \rightarrow \text{LMW component.}$$

The 7S protein reaches a maximum at 1·5 M GuHCl and is absent at 3 M GuHCl concentration (figure 2b). Also at 3 M GuHCl, most of the LMW component (nearly 90 %) is already formed and has an $S_{20,w}$ value of 2S. At 4 M GuHCl the pattern consists of 2S protein only. However, with α-globulin from sesame seed, Prakash and Nandi (1977) have reported that above 1·5 M GuHCl most of the protein was present as 2S component. These results indicate that GuHCl is more effective than urea in dissociating the protein.

Effect of SDS

In figure 1D, are shown the velocity sedimentation patterns of glycinin in SDS solutions of 0·7 mM and 3·5 mM concentrations. Here the protein appears to dissociate directly to the 2S protein (figure 1D and figure 2c).

$$11S \rightarrow 2S.$$

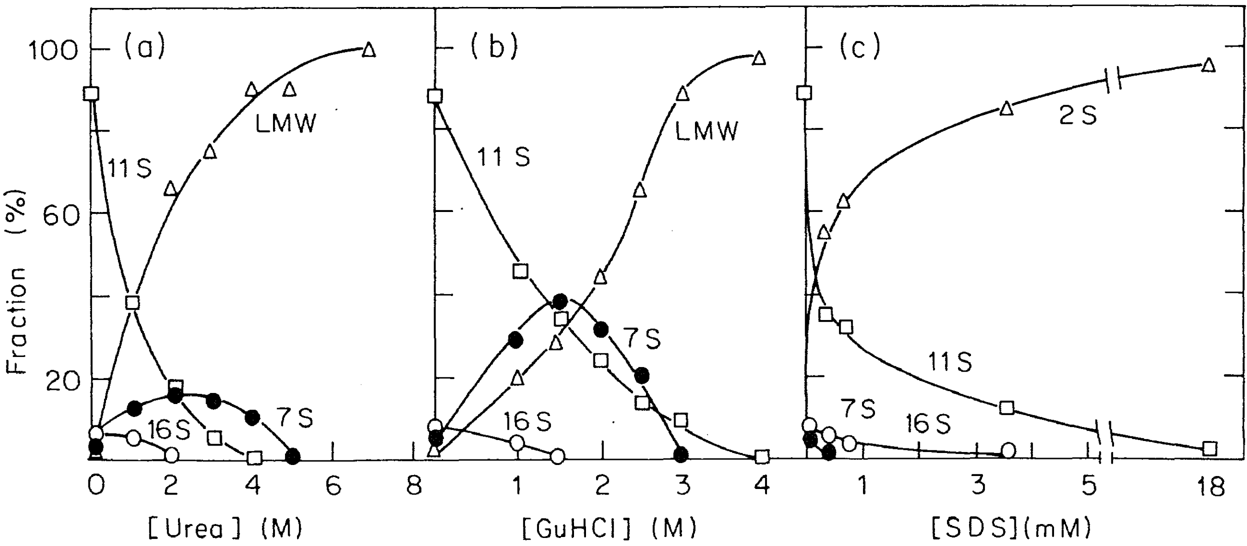

Figure 2. The proportion of various protein fractions as a function of (a) urea (b) GuHCl and (c) SDS concentrations. The various fractions are indicated in each figure.

unusual. SDS is known to be a more powerful dissociating and denaturing reagent than urea or GuHCl. Gururaj Rao and Narasinga Rao (1979) have in their study of the effect of SDS on the high molecular weight protein of mustard and rapeseed have reported that the protein directly dissociates to the 2S component. However, α-globulin, does not dissociate directly to the 2S protein in SDS solutions. 7S and 4S intermediates in both urea and GuHCl solutions were observed (Prakash and Nandi, 1977).

Discussion

A comparison of the effect of urea, GuHCl and SDS on glycinin, shows the following. SDS is more effective than GuHCl and in turn GuHCl is more effective than urea in dissociating the protein. The order is

$$\text{SDS} \gg \text{GuHCl} > \text{Urea}.$$

Meyer and Kauzmann (1962) have reported that unlike urea and GuHCl, detergents do not compete for peptide hydrogen bonds, but instead weaken the hydrophobic bonds. These results suggest the possibility of the predominance of hydrophobic bonds and ionic linkages over hydrogen bonds in the stabilization of the native structure of glycinin. This strong dissociative action of SDS on glycinin may be due to its greater ability to break hydrophobic bonds and ionic linkages (Jencks, 1969).

The 7S protein completely dissociated at 4 M urea and 3 M GuHCl at which concentration the 11S protein is still present. These results suggest that (i) the subunits of 7S component are not held predominantly by hydrophobic bonds and (ii) the conformational stability of 7S protein is low compared to the 11S protein. These results are in conformity with the results of Robert and Briggs (1963) and Wolf *et al.* (1964) who have studied the effect of alcohols on glycinin. Prakash *et al.* (1980) have reported similar results in their study of the effect of SDS, urea and GuHCl on the conformation of α-globulin.

A comparison of the per cent fraction of 7S component both in urea and GuHCl solutions shows (figure 2a, b) that the maximum proportion of 7S component is $\sim 16\%$ in 2 M urea and $\sim 39\%$ in 1·5 M GuHCl. This suggests that the sequence of dissociation in urea and GuHCl could be different due to differences in the mechanism of their action. Alternatively urea may have a greater penetration in to the interior of the protein molecule as compared to GuHCl (Hibband and Tulinsky, 1978).

The $S_{20,w}$ value of different protein fractions decreases with the increase in denaturant concentrations. This decrease possibly reflects the extent of denaturation (Holcomb and Vanholde, 1962). Figure 3 shows a plot of $S_{20,w}$ value of glycinin against denaturant concentration till it is completely dissociated. The $S_{20,w}$ value of 11·5 in native state decreases only very slightly in SDS solution. In 18 mM SDS solution, the $S_{20,w}$ is 10·9 and the maximum change in $S_{20,w}$ value is observed between zero and 3·5 mM SDS concentration. With the increase in urea and GuHCl concentration the $S_{20,w}$ value of the protein decreases. Greater change is observed in GuHCl solution. The value of 7·8S in 2·5 M GuHCl is almost equal to that in 5 M urea. This may reflect

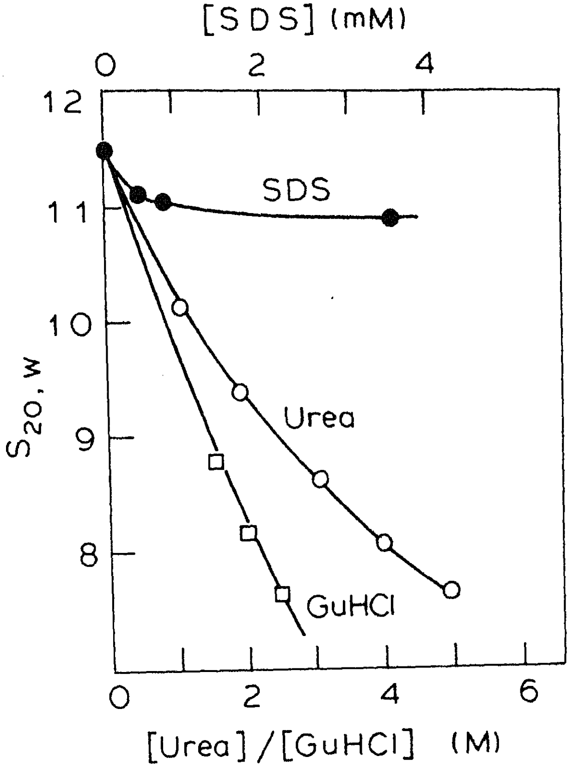

Figure 3. $S_{20,w}$ value of glycinin as a function of denaturant concentration.

molar concentration (Tanford, 1968, 1970; Green and Pace, 1974). The $S_{20,w}$ value of 7S protein also decreases with increase in denaturant concentration. Analysis of 2S protein has not been possible because of the very diffuse peaks obtained with the protein.

When studying the effect of reagents such as urea, GuHCl and SDS on multimeric proteins one faces the question whether dissociation and denaturation reactions occur sequentially or simultaneously? Very often it is difficult to get a clear answer. In this investigation we have studied the effect of urea, GuHCl and SDS on glycinin by sedimentation velocity techniques. The dissociation of glycinin by these reagents is clearly seen by the appearance of components of low $S_{20,w}$ value.

In the case of homogeneous proteins, $S_{20,w}$ value of the protein decreases when it is denatured (Wales and Coll, 1969). Determination of the $S_{20,w}$ value of the peak of undissociated glycinin at various concentrations of urea, GuHCl or SDS shows that it decreases considerably. This is also supported by secondary structural changes in the

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Effect of Centchroman (67/20) and 78/224 on nucleic acid and protein biosynthesis during implantation in rats

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Abstract. The biosynthesis of nucleic acids and proteins was studied in rat uterus by following the incorporation of [^{3}H]-thymidine, [^{3}H]-uridine and [^{14}C]-leucine in control and pregnant rats in the presence and absence of two anti-implantation drugs. One of the drugs, 78/224 caused a significant increase in incorporation whereas the other drug, Centchroman, caused an inhibition in incorporation of all the three precursors. The implications of these changes in the light of estrogenicity, agonist and antagonist actions of anti-estrogens have been analysed. The importance of homeostatic mechanisms involved in nucleic acids and proteins for the maintenance of constant internal milieu for blastocyst attachment has been discussed.

Keywords. Nucleic acid and protein synthesis; implantation; Centchroman.

Introduction

Implantation of rat blastocyst with the uterine endometrium is an inextricable phenomenon brought about by an interaction of the two steroidal hormones estrogen and progesterone with the target tissues. The mechanism by which the requisite changes are brought about is not known. When RNA extracted from the uteri of estrogen (E) stimulated rats was administered to E deprived ovariectomised rats, the resultant morphological changes in treated uteri were similar to those that occur following the administration of E (Segal *et al.*, 1965; Nishikawa, 1978; Lejeune *et al.*, 1982). Therefore, in the process of implantation, nidatory E may first promote RNA synthesis in the endometrium and then exhibit its action through the medium of RNA thus synthesised. Thus, a precise amount of nucleic acid and protein synthesis is an essential need of implantation. Any deviation from it leads to anti-implantation and anti-fertility activities.

Interestingly, both anti-estrogenic and estrogenic agents alter the preparatory changes necessary for implantation. Two anti-implantation agents synthesised in Central Drug Research Institute, Lucknow *viz.*, 78/224 (1,2-diethyl-1-3-bis-(*p*-

was of interest to study the synthesis of nucleic acid and proteins in the rat uterus during days 2, 5 and 6 of pregnancy under conditions of drug treatment.

Materials and methods

The radioactive precursors, thymidine (methyl-T), (45 mCi/m mol), [^{3}H]-uridine (12·7 mCi/m mol) and [^{14}C]-leucine (41·85 mCi/m mol), were obtained from Bhabha Atomic Research Centre, Bombay.

Virgin female albino rats (Wistar strain) bred in the colony of our Institute (weight 125 ± 5 gm) were kept in uniform animal husbandry conditions in air-conditioned quarters. Females were caged overnight with proven males in 3:1 ratio and next morning their vaginal smears were examined. The detection of sperm was used to compute the first day of pregnancy. Implantation and inter-implantation sites were differentiated on the 6th day of pregnancy by an intravenous injection of 1 ml of 1 % solution of pontamine Blue by a 27 gauge needle 1 h before sacrificing. Animals were administered orally drug no. 78/224 at the dose of 0·25 mg/rat for 5 days and the other drug Centchroman at the dose of 0·05 mg/rat. Rats were sacrificed by decapitation and uteri were immediately taken out, blotted, weighed, chopped into pieces and then processed. In each group 6 rats were undertaken.

[^{3}*H*]-*Thymidine incorporation*

The incorporation of the precursor into DNA was studied by the method of Ledford *et al.* (1978) with slight modifications and DNA extraction carried out by the method of Burton (1956). [^{3}H]-Thymidine (10 μCi) was incubated with pieces of each uterus in Kreb's Ringer Bicarbonate buffer at 37°C in a metabolic shaker for 1 h. Incubation was stopped by chilling. Each tissue sample was then washed thrice with cold buffer. Tissues were homogenized in an all glass homogenizer in Tris buffer; pH 7·4, at 0°C.

Homogenates were centrifuged at 800 *g* for 10 min in cold and the supernatant was discarded. The pellets were extracted with 1 ml of 0·6 N cold perchloric acid (PCA) and then washed consecutively with 2·0 ml of absolute alcohol, 2:1 mixture of chloroform; methanol and then with cold solvent ether. The pellets were then dissolved in 3 ml of 0·2 N NaOH and incubated at 37°C for 16 h and then once again 1·0 ml of cold 0·6 N PCA was added. Pellets were treated twice with 0·2 N PCA at 70°C for 10 min and then centrifuged. The supernatant was taken to represent the DNA digest. Aliquots were taken for radioactivity and A_{260} measurements. Calf thymus DNA (Sigma) was used as standard.

[^{3}*H*]-*Uridine incorporation into RNA*

This was carried out as described by Singh and Roy (1979).

[^{14}*C*]-*Leucine incorporation into protein*

This was done following the procedure described by Roy *et al.* (1978).

Protein content was measured by Lowry's method (1951).

Results

The results obtained on the synthesis of DNA, RNA and protein are given in tables 1, 2, and 3. The tables clearly show that the DNA synthesis on the 5th day of pregnancy *i.e.*, on the day of implantation was 5·3 fold more than the second day of pregnancy ($P < 0·01$). The rate of DNA synthesis in the implantation sites was 3·3 fold more than in the inter-implantation sites ($P < 0·01$). The synthesis of DNA on 5th day of

Table 1. Effect of Centchroman and 78/224 on DNA synthesis in rat uterus during implantation.

S. No.	Group	[^{3}H]-Thymidine incorporation (cpm/μg DNA)
1.	Intact control	2·01 ± 0·02
2.	2nd day of pregnancy	23·72 ± 1·60
3.	5th day of pregnancy	126·51 ± 12·34
4.	6th day of pregnancy (implantation site)	146·20 ± 2·0753
5.	6th day of pregnancy (inter-implantation site)	43·80 ± 0·9173
6.	5th day of pregnancy (78/224 treated)	158·09 ± 5·71
7.	5th day of pregnancy (Centchroman treated)	52·25 ± 9·13

Values are mean of 6 animals ± S.E.
Data were analysed through Student's '*t*' test and '*f*' test.
1 *vs* 2, $P < 0·01$; 1 *vs* 3, $P < 0·01$; 2 *vs* 3, $P < 0·01$; 4 *vs* 5, $P < 0·01$; 3 *vs* 6, $P < 0·05$; 3 *vs* 7, $P < 0·01$.

Table 2. Effect of Centchroman and 78/224 on RNA synthesis in rat uterus during implantation.

S. No.	Group	[^{3}H]-Uridine incorporation (cpm/μg RNA)
1.	Intact control	2·35 ± 0·01
2.	2nd day of pregnancy	4·15 ± 0·0509
3.	5th day of pregnancy	15·01 ± 1·2281
4.	6th day of pregnancy (implantation site)	25·95 ± 1·746
5.	6th day of pregnancy (inter-implantation site)	8·10 ± 0·7179
6.	5th day of pregnancy (78/224 treated)	43·43 ± 6·8199
7.	5th day of pregnancy (Centchroman treated)	3·28 ± 1·5502

Values are mean of 6 animals ± S.E.
Data were analysed through Student's '*t*' test and '*f*' test.

Table 3. Effect of Centchroman and 78/224 on protein synthesis in rat uterus during implantaion.

S. No.	Group	$[^{14}C]$-Leucine incorporation (cpm/mg protein)
1.	Intact control	6·66 ± 1·23
2.	2nd day of pregnancy	29·83 ± 1·92
3.	5th day of pregnancy	123·80 ± 13·7827
4.	6th day of pregnancy (implantation site)	179·98 ± 12·2684
5.	6th day of pregnancy (inter-implantation site)	124·22 ± 5·6333
6.	5th day of pregnancy (78/224 treated)	159·28 ± 1·544
7.	5th day of pregnancy (Centochroman-treated)	47·28 ± 2·3088

Values are mean of 6 animals ± S.E.
Data were analysed through Student's '*t*' test and '*f*' test.
1 *vs* 2, $P < 0·01$; 1 *vs* 3, $P < 0·01$; 2 *vs* 3, $P < 0·01$; 4 *vs* 5, $P < 0·01$; 3 *vs* 6, $P < 0·05$; 3 *vs* 7, $P < 0·01$.

pregnancy after treatment with the estrogenic drug (18/224) was 6·7 fold and 1·2 fold more than the values obtained on the 2nd and 5th day of normal pregnancy, respectively ($P < 0·05$). In the case of Centchroman treated rats the rate of synthesis was slightly more than that obtained on the second day of pregnancy but decreased by more than two fold when compared to the values obtained on the fifth day of normal pregnancy.

RNA synthesis on the 5th day of pregnancy was 3·6 fold more than that obtained on the second day ($P < 0·01$), while synthesis on the 6th day in the implantation sites was 3·2 fold more than in inter-implantation sites ($P < 0·01$). The RNA synthesis of estrogenic drug (18/224) treated rats was 2·9 fold and 10·5 fold more than the values obtained on the 5th day and 2nd day of normal pregnancy, respectively ($P < 0·01$). The RNA synthesis on the 5th day of pregnancy treated with Centchroman was 5-fold less than that seen with the normal 5th day of pregnancy.

Protein synthesis on the 5th day of pregnancy was 4-fold more than on the second day of pregnancy. An increase of 1·4 fold was observed in the implantation sites compared to the inter-implantation sites. In propene treated rats, protein synthesis was 1·25 fold more than that seen on 5th day of pregnancy. After Centchroman treatment the rate of protein synthesis was 2·5 fold lower than that of 5th day of normal pregnancy. The data were analysed by Student's '*t*' test and '*f*' test and found to be significant at 1% level.

Discussion

Estrogenic and antiestrogenic drugs are good probes for studies on hormonal *et al.*, 1983; Jordan *et al.*, 1981; Jordan, 1982; Jordan and Gosden, 1982, 1983). A similar side chain is present in tamoxifen, thioxifene and also in our compound Centchroman. A *cis-trans* isomerism is very common in many of the non-steroidal antiestrogens. Interestingly, the *cis*-isomers possess estrogenic while *trans* isomers show anti-estrogenic response. The present compound Centchroman also shows *cis-trans* isomerism.

For most of the period the uterus remains in refractory state and it does not permit the blastocyst to implant. Sensitivity is brought about by the action of steroidal hormones (Sutherland and Murphy, 1982).

Throughout the preimplantation period, mouse uterine glands are active secretory structures similar to other exocrine cells. Thus, the uterine gland of mouse must be considered a source of uterine fluid proteins at the time of implantation. These uterine proteins help in the attachment of blastocyst. The inability of blastocyst attachment with the uterine endometrium due to anti-implantation agents could be due to altered level of synthesis of nucleic acids and proteins which is an essential requirement for the implantation process (Randall and Enders, 1981).

Antiestrogens have been found to show both agonist and antagonist properties. Roy and Datta (1976) and Durrani *et al.* (1979) have observed that Centchroman exerts a very weak estrogenic action in the absence of E, whereas in the presence of potent E, it exerts a relatively strong anti-estrogenic action. Thus, the decreased synthesis of nucleic acid and protein in the presence of Centchroman might be due to its antagonistic action. The observations of the present work are comparable to that of Reid and Heald (1971). The above decrease may alter the epithelial morphology which may be a factor in its post-coital contraceptive action (Poteat, 1981).

The increase in DNA synthesis due to the action of estrogens, similar to the present findings, has been observed by many workers (Shelesnyak and Tie, 1963; Miller *et al.*, 1968; Mohla *et al.*, 1970; Heald and O'Grady, 1970; Reid and Heald, 1971; Ledford *et al.*, 1978; Harris and Gorski, 1978). An increase in the synthesis of nucleic acids and proteins by 78/224 might be due to an altered internal milieu in uterine luminal secretions and in uterine epithelial cells which become hostile for attachment of blastocyst. The present work is also in agreement with the estrogenic nature of drugs as reported earlier by Prakash and Roy (1980, 1981, 1982). Thus, from the present work the importance of homeostatic mechanisms involved in nucleic acids and protein metabolism, an essential pre-requisite for implantation process is well evident. Both increase and decrease in the synthetic and degradative processes disturb the uterine internal milieu which leads to anti-implantation and anti-fertility activities.

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Sharing of antigens among filarial species revealed by antibody dependent cell-mediated reactions†

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Abstract. Antisera raised in albino rats against microfilariae of *Litomosoides carinii, Brugia pahangi, Brugia malayi* and sera from *Bancroftian elephantiasis* patients promoted rat neutrophil-mediated adherence and cytotoxicity to the microfilariae. Pre-treatment of the immune sera, with microfilarial antigen at a final concentration of 5 and 25 μg per ml blocked cellular adherence and cytotoxicity to the microfilariae indicating the presence of cross-reactive antibodies. The heterologous immune sera were effective in eliminating the circulating *Litomosoides carinii* microfilariae in *Mastomys natalensis.*

Keywords. Microfilariae; antigen; cross-reaction; antibody-dependent cellular cytotoxicity.

Introduction

The onset of an antibody that promoted cell-mediated killing of microfilariae is associated with the termination or reduction of circulating microfilariae in albino rats infected with *Litomosoides carinii* (Subrahmanyam *et al.*, 1976), in hamsters with *Dipetalonema viteae* (Weiss, 1978), in cats with *Brugia pahangi* (Ponnudurai *et al.*, 1974), in rhesus monkeys with *B. malayi* (Aiyar *et al.*, 1982) and in humans with *Wuchereria bancrofti* (Mehta *et al.*, 1981).

The antibodies seem to be directed to the sheath and/or cuticle of the parasites. There is some evidence to indicate that certain antigenic determinants are shared between different filarial parasites. The present report further substantiates the sharing of antigens between *L. carinii, B. pahangi, B. malayi* and *W. bancrofti* as revealed by the antibody-dependent cell-mediated cross reactions.

Materials and methods

Filarial infections

Rats were infected with *L. carinii* by infective mites, *Bdellonyssus bacoti.* Methods for the maintenance of the infection and monitoring it by means of microfilarial counts in

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B. pahangi and *B. malayi* infections in *Mastomys natalensis* ("GRA" strain) were maintained as described by Sanger *et al.* (1981).

Media

RPMI-1640 [RPMI-1640 (GIBCO) was buffered with HEPES (25 mM)], MEM/FCS/DNase (minimum essential medium (MEM) (GIBCO) was supplemented with 10% foetal calf serum (FCS) (GIBCO) inactivated at 56°C for 1 h. DNase (SIGMA) was added to the medium at a concentration of 30 mg/L) and Tryode/Gelatin/DNase (tyrode solution was prepared with 0·1% gelatin and 30 mg/L of DNase) were supplemented with penicillin (100 I.U./ml) and streptomycin (100 μg/ml) and used. All media were sterilised by passing through 0·22 μm millipore filter unit (Millipore Corp., Bedford, USA) and stored at 4°C until use.

Microfilariae

Microfilariae (MF) of *L. carinii* (Lc-Mf), *B. pahangi* (Bp-Mf) and *B. malayi* (Bm-Mf) were isolated from the blood of infected *Mastomys natalensis* by density gradient centrifugation over iso-osmotic Percoll® (Pharmacia) as described by Chandrashekar *et al.* (1984).

Microfilarial antigens

The microfilariae were sonicated in PBS at 4°C (20 KHz; 8 × 30 sec). The sonicated preparation was spun at 20,000 rpm for 30 min at 4°C. The supernatant from each fraction was dialysed and the protein content determined (Bradford, 1976) and stored in suitable aliquots at −70°C.

Sera

Antisera were prepared from the blood of 4–6 weeks old male albino rats ("Wistar" strain) immunised with sonicated Mf (actively immunised rat serum, AIRS). Rats were immunised with three intramuscular injections, 10–15 days apart of microfilarial sonicates ($1{\cdot}8-2{\cdot}5\times10^5$ Lc-Mf or $0{\cdot}5-1{\cdot}0\times10^5$ Bp-Mf or Bm-Mf), emulsified in Freund's complete adjuvant, and bled 2–3 weeks after the last booster dose. Normal rat serum (fNRS) was freshly prepared before each experiment. The blood drawn by cardiac puncture, was allowed to clot at 37°C for half an hour before the serum was separated by centrifugation at 2000 *g* for 10 min at 4°C. Sera from patients with elephantiasis due to *Wuchereria bancrofti* (ELS) were isolated from venous blood.

Cells

Neutrophils: Peritoneal exudate cells (PEC) were obtained from two months old albino rats, 16–20 h after intraperitoneal injection of 5 ml of 12·5% casein. The peritoneal cavities were washed out with 20 ml of MEM containing 10 units/ml of heparin. The cells were washed twice with MEM/FCS/DNase and finally suspended in

Eosinophils

PEC obtained from normal rats, 48–72 h after intraperitoneal injection of 5 ml of paraffin oil, were washed with MEM/FCS/DNase and layered over metrizamide gradients. Eosinophils of greater than 95 % purity were obtained at 24/26 metrizamide interface.

The viability of the cells was assessed by trypan blue exclusion technique.

Treatment of immune sera

Lc-Mf AIRS, Bp-Mf AIRS, Bm-Mf AIRS and ELS were treated with microfilarial antigen. For this purpose, 1 ml of each of the immune serum was separately treated with either Lc-Mf antigen, Bp-Mf antigen or Bm-Mf antigen taken in 1 ml of RPMI-1640 medium at a final concentration of 5 and 25 μg of antigen per ml. The mixture was incubated at 4°C overnight and was then spun at 20,000 rpm. The supernatant was used in the ADCC assay. The control sera were similarly diluted with 1 ml RPMI-1640.

Culture conditions and cell-adherence assay

Fifty μl of RPMI containing Mf (100 of *B. pahangi* or *B. malayi* or 200 of *L. carinii*) were incubated with 50 μl of cells (5×10^5) and 50 μl of fNRS and/or AIRS in a final volume of 0·2 ml in a flat bottom 96 well-microtiter plate (Costar, Massachusetts, USA). The plates were incubated in an atmosphere of 5 % CO_2, 95 % air for 4–16 h at 37°C. After incubation, the samples were examined microscopically for cellular adherence and cytotoxicity to Mf as described by Mehta *et al.* (1981).

Passive transfer experiment

Three to four weeks old male *Mastomys natalensis* were used in this experiment. Five groups, each comprising 6 animals, were given intraperitoneal injection of $1{\cdot}25 \times 10^6$ Lc-Mf suspended in RPMI-1640. The microfilaremia was monitored (as described by Bagai and Subrahmanyam, 1968) for 3 days. On day 3, 4 groups were treated intraperitoneally with 1·5 ml of Lc-Mf AIRS, Bp-Mf AIRS, Bm-Mf AIRS and ELS respectively. The 5th group was given fNRS that served as the control group. After treatment, the microfilaremia was monitored till day 14 in all groups.

Results

Cell-mediated adherence and cytotoxicity to Mf with different immune sera

L. Carinii Mf: ADCC assay was carried out with *L. carinii* Mf in the presence of fNRS, Lc-Mf AIRS, Bp-Mf AIRS, Bm-Mf AIRS and ELS, using neutrophils and eosinophils as effector cells. The data are presented in table 1. As seen from the table 1, except fNRS, the rest of the sera promoted significant cellular adherence and cytotoxicity to the Mf. However the cytotoxic effect due to ELS was comparatively very low. Eosinophils,

Cells	%	fNRS	Lc-Mf AIRS	Bp-Mf AIRS	Bm-Mf AIRS	EES
Neutrophils	Adherence	0	100	100	100	73±3
	Cytotoxicity	0	76±3	71±2	75±10	14±2
Eosinophils	Adherence	0	100	100	100	76±4
	Cytotoxicity	0	7±2	2±1	16±2	7±2

Mean ± SEM of 3 experiments.

although readily adhering to the Mf, did not exert much cytotoxicity to the parasites.

Brugia Mf: The results with Mf from *B. pahangi* and *B. malayi* were similar to those obtained with *L. carinii* (data not shown) except that fNRS alone promoted some adherence of cells without any apparent cytotoxicity to the parasites. Homologous sera as well as Lc-Mf AIRS were equally potent in the ADCC reaction.

ADCC inhibition assay

To ascertain the extent of cross reaction, the immune sera were pretreated with either of the 3 microfilarial antigens, Lc-Mf, Bp-Mf and Bm-Mf at a final concentration of 5 and 25 μg of antigen/ml as described under 'methods'. The treated sera were then used in the ADCC reaction against microfilariae of the 3 species using neutrophils as effector cells. The sera were found to markedly inhibit ADCC to the microfilariae (figure 1) at an

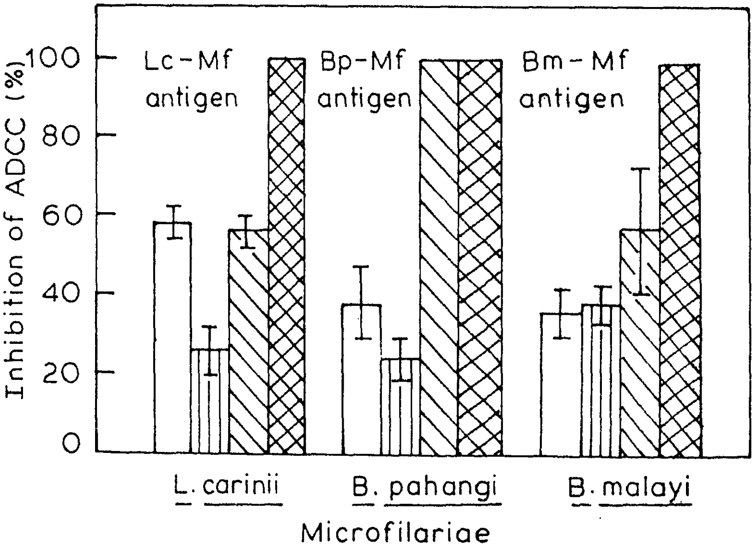

Figure 1. Inhibition of ADCC against microfilariae of different species of immune sera raised against *L. carinii*, □; *B. Pahangi*, ▥; *B. malayi*, ▧; and of sera from bancroftian elephantoid patients ▩; on absorption with microfilarial antigens.

antigen concentration of 5 μg/ml. The ADCC was completely blocked when the sera were treated with 25 μg of antigen, indicating the extensive sharing of antigens among these species. The low cytotoxicity of ELS was abolished on prior treatment with 5 μg of antigen.

Effect of antisera on circulating microfilariae of L. carinii

The effect of different antisera on circulating *L. carinii* microfilariae *in vivo* was studied by a passive transfer experiment described under 'methods'. An intraperitoneal injection of $1 \cdot 25 \times 10^6$ of *L. carinii* microfilariae in a 3–4 week old *Mastomys* resulted in a parasite count of about 200 Mf/10 μl of the peripheral blood (table 2). The microfilaremia was stabilised in the animals usually at day 3 when the animals were given intraperitoneally 1·5 ml of the immune sera. There was a rapid disappearance of the microfilariae from the circulation within 24 h (table 2) in all the groups except the one receiving fNRS. The parasites did not reappear till day 14 indicating that the immune sera killed the circulating Mf *in vivo*.

Table 2. Effect of heterologous immune sera on circulating microfilariae of *Litomosoides carinii* in *Mastomys natalensis*.

	Microfilaremia in 10 μl of blood ($x \pm$ S.D.)					
Group†	Day 1	Day 3	Treatment with	Day 4	Day 7	Day 14
1	110 ± 44	171 ± 53	Lc-Mf AIRS	0	0	0
2	80 ± 31	164 ± 52	Bp-Mf AIRS	1 ± 0·9	0	0
3	91 ± 23	188 ± 45	Bm-Mf AIRS	2 ± 1	0	0
4	193 ± 42	180 ± 39	ELS	0	0	0
5	78 ± 21	241 ± 58	fNRS	247 ± 60	273 ± 97	295 ± 100

† Each group consisted of 6 *Mastomys*.

Discussion

There is increasing evidence on sharing of antigens among filarial parasites. Neppart (1974) found cross reactions between *Onchocerca* Sp., *Dirofilaria immitis* and *L. carinii* in the closed hexagonal immunodiffusion assay. The antigens of *Setaria digitata* react with serum antibodies of *W. bancrofti* infected subjects in ELISA (Dissanaike and Ismail, 1980). Sharing of antigens between *L. carinii* and *W. bancrofti* has also been demonstrated (Rao *et al.*, 1980). Tandon *et al.* (1983), based on the presence of common antigens between *L. carinii*, *D. viteae*, *B. malayi* and *B. pahangi* parasites, studied the pattern of antibody titres in sera of animals infected with these parasites using *L. carinii* the ability of *L. carinii* antigens to neutralize the ADCC activity of different antisera against the microfilarial species and the ability of the antisera raised against these parasites and of ELS in clearing circulating *L. carinii* microfilariae from *Mastomys.* The antisera directed against *L. carinii*, *B. pahangi* and *B. malayi* was more active in inducing ADCC to these parasites than ELS. This difference may be due to the high titre of the antibodies in immunized rodents when compared to the antibodies resulting from infection in patients (ELS).

Acknowledgement

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Salt mediated changes in some enzymes of carbohydrate metabolism in halotolerant *Cladosporium sphaerospermum*

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Abstract. *Cladosporium sphaerospermum*, isolated from salt pans was halotolerant. When grown in the presence of salt, the activities of invertase, isocitrate lyase, fructose-1,6 diphosphate aldolase and malate dehydrogenase were found to be increased and that of amylase decreased. Both, enzyme activation as well as an increase in *de novo* synthesis of enzymes were found to be some of the mechanisms of salt mediated changes. This may be one of the adaptive mechanisms, in halotolerant *Cladosporium sphaerospermum.*

Keywords. *Cladosporium sphaerospermum*; halotolerant; invertase; amylase; aldolase; malate dehydrogenase; isocitrate lyase.

Introduction

The enzymes from extremely halophilic bacteria represent a fascinating example of adaptation. These enzymes perform their functions *in vivo* at 4–5 M NaCl, losing activity rapidly when exposed to low salt concentration (Lanyi, 1974). There is paucity of information regarding the salt effects in marine fungal systems as compared to bacteria.

Physiological studies on marine fungi have tended to concentrate mainly on their salinity requirements (Harrison and Jones, 1974), spore germination (Byrne and Jones, 1975), sporulation (Harrison and Jones, 1975), and some aspects of physiology and biochemistry (Jennings, 1983). Not much is known about the effect of salt on enzymes from fungal systems. The present study therefore deals with the effect of salt on some of the enzymes of carbohydrate metabolism in the fungus *Cladosporium sphaerospermum* isolated from salt pans.

Materials and methods

Isolation of fungal culture

The fungal culture was isolated by streaking the soil samples obtained beneath the salt

The identification of fungal culture was done with the kind help of Commonwealth Mycological Institute, Ferry Lane, Kew, Surrey, England.

Composition of liquid growth medium

The composition of the liquid growth medium employed was as follows (g/litre): Yeast extract 1·0; $(NH_4)_2SO_4$ 3·5; KH_2PO_4 10; $MgSO_4$ 2·0; Sucrose 85; Casamino acids 1·0; $CaCl_2$ 0·075; $ZnSO_4$ 0·010; $MnCl_2$ 0·005; ammonium molybdate 0·002; $Na_2B_4O_7$ 0·002 and $FeSO_4$ 0·002. The pH was adjusted to 5·5.

For experiments on amylase production, 1 % starch was used as a carbon source in the liquid growth medium instead of sucrose.

Growth of the culture in liquid medium

The culture was grown in 50 ml liquid medium in 250 ml Erlenmeyer flasks on a rotary shaker (180 rpm) at 30°C for 72 h unless and otherwise stated. The mycelia were harvested by filtration and rapidly transferred to -5°C before use. Cycloheximide whenever required was added at 10 μg/ml.

Method for the preparation of cell-free extract

For intracellular enzyme assays, a cell-free extract was prepared in 0·05 M Tris HCl buffer of pH 7·2 by grinding the frozen mycelia with a pestle in a chilled mortar with glass powder. The extract was centrifuged at 15,000 *g* for 30 min and the supernatant obtained was used for intracellular enzyme assays.

Assay method for FDP aldolase (fructose-1,6 diphosphate D-glyceraldehyde-3 phosphate lyase, EC 4.1.2.13)

FDP aldolase was assayed according to the method of Jagannathan *et al.* (1954). The unit of activity is expressed as the amount of enzyme which causes a change of 0·001 O.D. at 240 nm per min under the experimental conditions.

Assay method for malate dehydrogenase (L-malate; nicotinamide adenine dinucleotide oxidoreductase, EC 1.1.1.37)

Malate dehydrogenase was assayed as described by Ochoa (1955).

A unit of malate dehydrogenase is defined as the amount of enzyme needed to bring about decrease of 0·001 O.D. at 340 nm per min at 30°C.

Assay method for isocitrate lyase (threo-D-isocitrate glyoxylate lyase, EC 4.1.3.1)

Isocitrate lyase was assayed according to the method of Dixon and Kornberg (1959). A unit is defined as the amount of enzyme which causes an increase of 0·001 O.D. at 324 nm per min at 30°C under experimental conditions.

Assay method for invertase (β-fructofuranoside fructohydrolase, EC 3.2.1.26)

Invertase activity was measured by estimating reducing sugars released as described by Bernfeld (1955) method. A unit is described as the amount of enzyme which causes the liberation of 1 μmol of reducing sugar at 37°C per h.

Assay method for amylase (1,4 D-glucan maltohydrolase, EC 3.2.1.2)

Amylase activity was measured according to the method of Bernfeld (1955).

A unit is described as the amount of enzyme which causes the liberation of 1 μmol of maltose at 37°C per h.

Assay method for protein

Protein was assayed according to the method of Lowry *et al.* (1951).

Results and discussion

C. sphaerospermum was isolated from salt pans. It did not show an obligatory requirement of salt for growth. The mold was thus found to be halotolerant. Mold could utilize glucose, sucrose or starch as carbon sources. Extracellular invertase activity was found to increase when sodium chloride was added to the growth medium upto 2 M concentration, while amylase activity decreased (table 1).

Table 1. Effect of sodium chloride added in the growth medium on the activities of enzymes from *C. sphaerospermum*.

NaCl (M)	Invertase	Amylase	Malate dehydrogenase	FDP aldolase	Isocitrate lyase
	Units/flask (50 ml)		(Units/mg protein)		
0·0	320	708	27	90	81
1·0	667	314	67	112	101
2·0	1372	231	65	261	244

FDP aldolase, isocitrate lyase and cytosolic malate dehydrogenase showed significantly higher activities in cultures grown in the presence of sodium chloride (table 1). Earlier, Baxter and Gibbons (1956) have shown that in certain halophilic bacteria, a number of enzymes like isocitrate dehydrogenase, succinate dehydrogenase, malate dehydrogenase, lactate dehydrogenase and cytochrome oxidase, required very high salt

1970, Dundas and Halvorson, 1966, Liebl *et al.*, 1969) but the influence of salt on the regulatory properties of these enzymes has not been extensively investigated.

The effect of NaCl was also seen *in vitro* on the activity of partially purified isocitrate lyase. Here also both K_m and $V_{\max}$ values were found to be increased. Similar results were obtained with malate dehydrogenase. Isocitrate lyase and malate dehydrogenase showed normal Michaelis–Menten kinetics. Thus in all the cases *i.e.* invertase, isocitrate lyase and malate dehydrogenase, both K_m and $V_{\max}$ values were found to be increased (table 2) in the presence of sodium chloride as compared to control.

Table 2. Effect of sodium chloride on K_m and $V_{\max}$ values of invertase, isocitrate lyase and malate dehydrogenase from *C. sphaerospermum*.

Enzyme	System	K_m (M)	$V_{\max}$
Invertase	Control	$2\cdot5 \times 10^{-5}$	0·91
	with NaCl	$7\cdot14 \times 10^{-3}$	2·5
Isocitrate lyase	Control	$1\cdot55 \times 10^{-5}$	38·4
	with NaCl	$1\cdot33 \times 10^{-4}$	333·0
Malate dehydrogenase	Control	5×10^{-5}	50·0
	with NaCl	$16\cdot6 \times 10^{-5}$	136·42

Sodium chloride (2M) was added *in vitro* to the assay mixture.

In order to investigate the effect of NaCl on the *de novo* synthesis of proteins *C. sphaerospermum* was grown in the normal medium for 24 h and transferred to medium containing sodium chloride and further incubated till 72 h in the presence and absence of cycloheximide. The activities of FDP aldolase, isocitrate lyase and malate dehydrogenase were found to increase in the absence of cycloheximide. But when a similar experiment was conducted in the presence of cycloheximide, there was no significant increase in the activity of isocitrate lyase suggesting that sodium chloride may be activating the *de novo* synthesis of this enzyme. Malate dehydrogenase and FDP aldolase however did not register similar effects under these conditions suggesting that sodium chloride may not be involved in activation of *de novo* synthesis of these enzymes. The activities of FDP aldolase and malate dehydrogenase were however found to be increased in the presence of cycloheximide, the reasons of which are not understood at present (table 3). This effect of cycloheximide in increasing the activities of some enzymes were shown earlier by us in *Neurospora crassa* (Shinde and Chhatpar, 1981).

At present we are not in a position to explain where and how much salt gets accumulated intracellularly. However, growing the fungus in the presence of different concentrations of salt was found to influence distinctly the activities of a number of enzymes.

grown in medium and transferred to medium in the presence of NaCl and with and without cycloheximide.

Growth conditions		Isocitrate lyase (units/mg protein)	Malate dehydrogenase (units/mg protein)	FDP aldolase (units/mg protein)
Initially grown on (for 24 h)	Transferred to (for further 48 h)			
Medium	No transfer	49·95	9·99	36·62
Medium + NaCl (1M)	No transfer	138·65	18·48	110·91
Medium	Medium + NaCl (1M)	255·14	15·00	131·32
Medium	Medium + NaCl (1M) + Cycloheximide	112·65	30·03	165·21

Several possible explanations can be given for these observations: (i) salt might have activated some enzymes post-translationally, (ii) salt might have increased the production of some enzymes by either increasing the rate of transcription or translation, (iii) both mechanisms might have been operating simultaneously for some enzymes, (iv) salt might have changed the turnover rates of enzymes. In our experiments sodium chloride was found to enhance the *de novo* synthesis of isocitrate lyase as well as activate the enzymes FDP aldolase, malate dehydrogenase, invertase and isocitrate lyase.

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Prediction and comparison of the secondary structure of legume lectins

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Abstract. Secondary structure prediction for the 4 legume lectins: Concanavalin A, soybean agglutinin, favabean lectin and lentil lectin, was done by the method of Chou and Fasman. This prediction shows that these four lectins fall into a structurally distinct class of proteins, containing high amounts of β-sheet and β-turns. There is a notable similarity in the gross structure of these proteins; all four of them contain about 40–50 % of β-sheet, 35–45 % β-turn and 0–10 % of α-helix. When the secondary structure of corresponding residues in each pair of these lectins was compared, there was a striking similarity in the Concanavalin A-soybean agglutinin and favabean lectin-lentil lectin pairs, and considerably less similarity in the other pairs, suggesting that these legume lectins have probably evolved in a divergent manner from a common ancestor. A comparison of the predicted potential β-turn sites also supports the hypothesis of divergent evolution in this class of lectins.

Keywords. Secondary structure prediction; legume lectins; divergent evolution; β-turns.

Introduction

Lectins are multivalent, carbohydrate binding and cell agglutinating proteins of plant and animal origin (Lis and Sharon, 1973; Goldstein and Hayes, 1978; Barondes, 1981). They elicit a variety of interesting cellular phenomena which include mitogenicity and preferential agglutination of cancerous cells (Rapin and Burger, 1974). In addition, lectins have been implicated in a variety of important functions in their parent organisms (Barondes, 1981).

The legume seeds have been found to be a rich source of lectins and for this reason the legume lectins have been the most widely studied. The legume lectins have also elicited a lot of interest due to the suggested evolutionary linkage between them (Hemperly and Cunningham, 1983). There are regions of extensive homology in the primary structure of these lectins (Hemperly and Cunningham, 1983; Foriers *et al.*, 1981). Secondary structure determination by circular dichroism (CD) reveals a considerable similarity in the sense that they all contain a large proportion of β-pleated sheet and negligible amount of α-helix (McCubbin *et al.*, 1971; Thomas *et al.*, 1979; Jirgensons, 1978). However, a detailed comparison of the secondary structure of these lectins, hitherto has not been done, though in one report the β-sheet regions of lentil lectin (LcL) were compared with those of Concanavalin A (Con A) (Foriers *et al.*, 1981).

Primary structure for 4 of the legume lectins *viz.*, Con A, soybean agglutinin (SBA),

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prediction of secondary structure and its comparison for these 4 lectins.

It may be noted that the secondary structure of Con A and LcL was predicted earlier (Chou and Fasman, 1974a; Foriers *et al.*, 1981). However, it became necessary for us to predict their secondary structure again since the above predictions were done with propensity values obtained from a smaller data base of protein structures, whereas in the present study propensities from a larger data base of protein structures is used. Another reason is that the prediction of β-turns in the case of Con A was not done in conjunction with other secondary structural types while the β-turns in LcL were not predicted at all.

Methods

Primary structures of Con A, LcL and Favin have been taken from the work of Edelman *et al.* (1972), Foriers *et al.* (1981) and Hopp *et al.* (1982). Secondary structure from X-ray crystal structure data for Con A has been obtained from Reeke *et al.* (1975). Amino acid sequence of SBA was obtained from the gene sequence data of Vodkin *et al.* (1983). Secondary structure prediction has been done by the method of Chou and Fasman (1978) using the propensity values P_α and P_β for the 20 amino acids derived from the crystal structure data of 29 proteins. β-Turns were predicted as reported by Chou and Fasman (1979a) using the tetrapeptide bend probability which is given by the expression,

$$P_t = f_i \times f_{i+1} \times f_{i+2} \times f_{i+3},$$

where f_i, f_{i+1}, f_{i+2} and f_{i+3} are the propensities of residues 1, 2, 3 and 4 of the tetrapeptide to be in position 1, 2, 3 and 4 of a β-turn. A cut off value of $P_t = 0{\cdot}75 \times 10^{-4}$ as recommended by Chou and Fasman (1978) was used. For all the tetrapeptides with $P_t > 0{\cdot}75 \times 10^{-4}$, the average propensities of α-helix ($\langle P_\alpha \rangle$), β-sheet ($\langle P_\beta \rangle$) and β-turn ($\langle P_t \rangle$) have been calculated. Tetrapeptides obeying the conditions $\langle P_\alpha \rangle < \langle P_t \rangle > \langle P_\beta \rangle$, and $P_t > 1{\cdot}00$ were assigned as β-turns. However, some of these turns were eliminated from the secondary structure assignment by considering them in conjunction with α-helical and β-sheet regions.

All the calculations in the present study were done on a Sord microcomputer (Sord Computer Systems Inc., Tokyo, Japan) using a programme* written in Basic.

For the sake of comparison the amino acid sequences of these lectins have been arranged as shown in figure 1 which is essentially the same as that reported earlier (Foriers *et al.*, 1981; Hemperly and Cunningham, 1983) excepting that no gaps were introduced in our comparison.

Residue to residue correlation analysis of the secondary structure has been carried out in the following way. The protein sequences have been aligned as shown in figure 1 and the secondary structure (predicted) of corresponding residues was checked for identity.

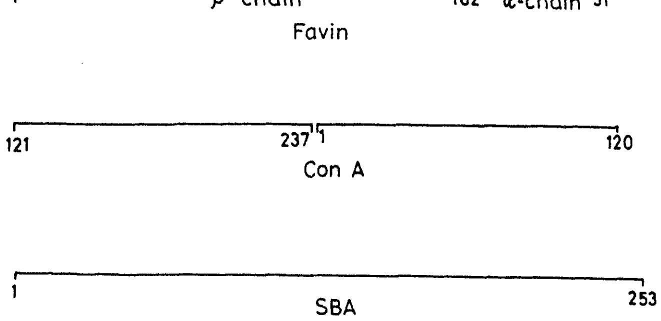

Figure 1. Alignment scheme for the 4 legume lectins; Con A, SBA, LcL and Favin, followed in the present study.

Results

The percentage content of the 3 secondary structural types (α-helices, β-sheets and β-turns) in the 4 legume lectins considered in the present study, *viz.* Con A, SBA, Favin and LcL, as predicted by the method of Chou and Fasman (1978) is given in table 1. For the sake of comparison, these structural details obtained from CD and X-ray crystallographic investigations are also given wherever available.

Details and comparative analysis of the various predicted α-helical and β-sheet regions of Con A, SBA, Favin and LcL, and these structural details obtained from X-ray crystallographic studies on Con A are given in table 2.

Table 1. Comparison of α-helix, β-sheet and β-turn content in legume lectins by different methods.

	Sheet (%)		Helix (%)		Turn (%)
Protein	Prediction	CD	Prediction	CD	Prediction
Con A	40·1 (38)	30	10·5 (2)	0	43 (39·2)
SBA	41·4	26	9·8	0	39·5
Favin β	45·6		7·1	0	37·9
α	56·9	—	13·7	0	19·6
LcL β	49·0		0	0	43·4
α	45·0	—	15·7	0	31·4

Values in parentheses indicate the results obtained from X-ray investigations.

Table 2. Comparison between secondary structural regions of legume lectins.

Con A				
X-ray	Predicted	SBA	Favin	LcL
	122–130*			
124–130				
		3–12	4–9	3–11
			18–22	15–20
139–143				
		23–28		
147–149				
			27–32*	
153–156				
	156–160	32–37	32–42	31–36
				43–48
		50–55	44–54	50–53
169–176,	172–181	57–61		
179–181				
				60–65
	182–191*	67–73*		
			64–72	68–72
188–200	192–199	74–80		
			84–89	84–91
208–215	210–216			
		90–95*		
			101–106	101–106
	228–233	107–112		
		117–121	113–121	115–120
4–11	3–9			122–128
		122–127*		
			136–140	137–141
23–30	25–32	141–149	144–148	146–150
		152–157*		
35–40				
			159–165*	
48–56	47–56	160–168		
			169–174	
59–66	60–67	174–181	177–181	
			1–6	1–10
73–79	72–79	189–196	9–16	12–16
81–85*	80–85*			
87–97	88–93	203–208	19–25	19–26
			30–36*	29–36*
103–106				
108–116	103–115	223–228	37–44	
		244–250		

Regions indicated with an asterisk (*) are α-helical; rest all are β-sheets. This table gives only α-helical and β-sheet regions in these lectins

Fasman (1978) is given in table 3. There are 38 turns in Con A, 33 turns in SBA, 26 turns in Favin and 27 turns in LcL. The β-turns of Con A, as obtained from crystal structure data (Reeke *et al.*, 1975), are also given in table 3.

Table 3. Predicted β-turn sites in legume lectins.

Lectin	β-Turn sites
Con A*	10–13, 14–17, 15–18, 28–31, 31–34, 34–37, 43–46, 55–58, 56–59, 67–70, 86–89, 97–100, 117–120, 134–137, 137–140, 143–146, 147–150, 149–152, 150–153, 160–163, 166–169, 183–186, 203–206, 216–219, 222–225, 226–229, 227–230, 229–232.
Con A	10–13, 12–15, 14–17, 16–19, 19–22, 34–37, 42–45, 56–59, 67–70, 71–74*, 75–78*, 94–97, 96–99, 102–105*, 116–119, 117–120, 119–122, 131–134, 134–137, 136–139, 142–145, 147–150, 149–152, 151–154, 160–163, 161–164, 164–167, 167–170, 169–172, 201–204, 203–206, 206–209, 216–219, 218–221, 222–225, 224–227, 225–228, 233–236.
LcL α-chain	26–29, 37–40, 40–43, 47–50
β-chain	4–7*, 12–15, 21–24, 22–25, 25–28, 27–30, 39–42, 54–57, 60–63*, 64–67, 66–69*, 74–77, 80–83, 92–95, 96–99, 97–100, 106–109, 108–111, 112–115, 131–134, 142–145, 151–154, 156–159
Favin α-chain	27–30, 46–49, 48–51
β-chain	5–8*, 13–16, 16–19, 22–25, 23–26, 55–58, 57–60, 74–77, 76–79, 80–83, 92–95, 96–99, 97–100, 105–108, 107–110, 109–112, 122–125, 130–133, 132–135, 141–144, 150–153, 155–158, 174–177.
SBA	5–8*, 15–18, 20–23, 28–31, 38–41, 42–45, 44–47, 46–49, 63–66, 80–83, 82–85, 86–89, 98–101, 104–107, 112–115, 114–117, 116–119*, 128–131, 130–133, 134–137, 136–139, 142–145*, 157–160, 169–172, 171–174, 181–184, 183–186, 185–188, 216–219, 229–232, 234–237, 236–239, 240–243.

β-Turn sites indicated with an asterisk (*) are eliminated in the assignment on comparison with propensities for helix and sheet in the neighbouring residues.

* β-Turns obtained from X-ray studies.

After aligning the 4 legume lectins as outlined in figure 1, secondary structure of corresponding residues in each pair, for all the 6 pairs was compared, and the results of such a comparison are presented in table 4. The correlations were calculated in percentages, with respect to the number of residues of the protein containing lesser number of residues in each pair. For example, in the Con A–Favin pair, there are 76 positions where the corresponding residues are in identical conformation, and the correlation is 32·6 % with respect to the number of residues in Favin. The same method is employed when residues of each structural type (namely, α-helix, β-sheet and β-turn) are compared.

Correlation analysis of β-turns is depicted schematically in figure 2. All the β-turns predicted using a cutoff value of $0{\cdot}75 \times 10^{-4}$ have been represented here. Two higher cutoff values of $1{\cdot}25 \times 10^{-4}$ and $2{\cdot}0 \times 10^{-4}$ have also been chosen for comparison purposes. The β-turns in two different proteins are considered to be corresponding

Table 4. Residue to residue correlation analysis of secondary structures of legume lectins.

Lectin pair	No. of corresponding residues in identical secondary structures			
	α-Helix	β-Sheet	β-Turn	Total
SBA–Con A	5 (20·8 %)	51 (57·3 %)	65 (65·0 %)	121 (51·05 %)
SBA–Favin	—	42 (44·2 %)	30 (37·9 %)	72 (30·9 %)
SBA–LcL	—	40 (42·1 %)	39 (46·4 %)	79 (37·6 %)
Con A–Favin	—	39 (43·8 %)	37 (46·8 %)	76 (32·6 %)
Con A–LcL	—	29 (32·6 %)	36 (42·9 %)	65 (30·9 %)
Favin–LcL	6 (85·7 %)	67 (69·8 %)	56 (66·67 %)	129 (61·4 %)

Values given in parentheses refer to per cent correlation calculated with respect to the number of residues of the protein containing lesser number of residues in each pair.

the lowest cutoff value, the correlation for all combinations is fairly high, with the highest values being associated with Con A–SBA and Favin–LcL pairs. This trend continues for the cases where higher cutoff values have been applied.

Discussion

The secondary structure content of the legume lectins (given as percentage α-helix etc., table 1) shows that there is a striking similarity in the gross structure of these proteins. All these proteins contain about 40–50 % β-sheet, 35–45 % β-turn and 0–10 % α-helix, and thereby fall into a structurally distinct class of proteins. The β-sheet, α-helical and β-turn content predicted here agrees well with X-ray structure determination of Con A by Reeke *et al.* (1975). So far, the structural studies on these lectins using CD, have only classified them as proteins containing a high amount of β-sheet and low amount of α-helix. This study shows that, in addition to these features, the legume lectins are also characterized by a high β-turn content.

Though the gross structure, as seen from table 1, is very similar for the 4 legume lectins considered in the present study, it is possible that the order in which the various secondary structural segments are arranged in each of them could be quite different, thereby leading to the possibility of each of them having a unique 3-dimensional structure of its own, which could be considerably different from that of the rest. In

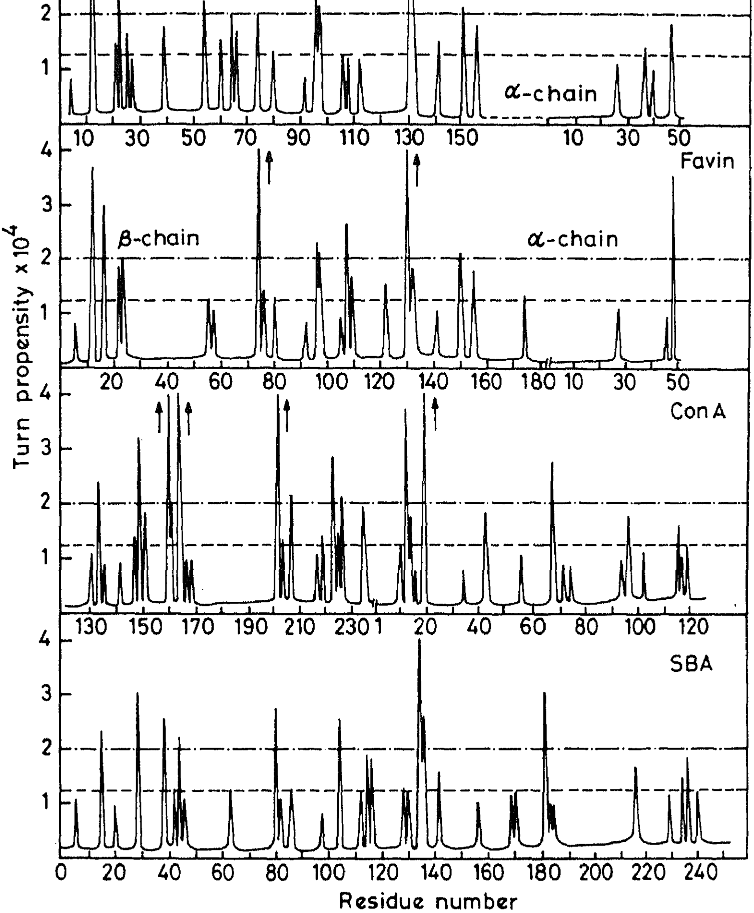

Figure 2. Propensities of β-turns in legume lectins. A cutoff value of $0{\cdot}75 \times 10^{-4}$ has been chosen to identify probable sites of β-turns which are indicated as sharp peaks. Two arbitrary cutoff values $1{\cdot}25 \times 10^{-4}$ (-------) and $2{\cdot}0 \times 10^{-4}$ (–·–·–·–) have been chosen for analysis purposes. Wherever the propensity is higher than $4{\cdot}0 \times 10^{-4}$ the peak is drawn up to $4{\cdot}0 \times 10^{-4}$ and is marked with an arrow. Dotted line in the figure corresponding to LcL denotes the missing stretch of 23 residues corresponding to residues 160–182 of Favin.

of the legume lectins are aligned, and two regions of similar secondary structure from different proteins have been considered to be correlating with each other, if, atleast half of the residues of the smaller segment are overlapping with the residues of the other. Such an analysis for the α-helical and β-sheet regions of the 4 lectins studied here is represented in table 2. There are 13 regions of overlap between LcL and Favin, 8 between SBA and Favin, 7 between SBA and LcL, 10 between SBA and Con A, 4

Table 5. Correlation analysis of the conservation of turns in various lectins pairs.

Lectin pairs	β-Turns conserved (%)		
	Cutoff values		
	$0{\cdot}75 \times 10^{-4}$	$1{\cdot}25 \times 10^{-4}$	$2{\cdot}0 \times 10^{-4}$
Con A–SBA	69·7 (23)	61·1 (11)	77·8 (7)
Con A–LcL	48·2 (13)	31·1 (6)	28·5 (2)
Con A–Favin	46·2 (12)	27·8 (5)	28·6 (2)
SBA–LcL	48·2 (13)	16·7 (3)	0·0 (0)
SBA–Favin	42·3 (11)	22·2 (4)	14·2 (1)
Favin–LcL	65·3 (17)	66·7 (12)	71·4 (5)

Values in the parentheses indicate number of correlating sites.
These percentages are calculations with respect to the protein containing lesser number of β-turns in each pair.

between Con A and LcL and 6 between Con A and Favin. The two highest correlation values belong to the Con A–SBA and Favin–LcL pairs, suggesting a closer similarity between Con A and SBA, and between Favin and LcL than in other pairs.

In order to investigate whether the above conclusion drawn from the comparison of the different secondary structural segments is correct, we have carried out a residue to residue comparison of the secondary structure of each pair of the legume lectins. The results of this analysis are given in table 4. The correlation for the total sequences in the pairs Con A–SBA and Favin–LcL is 51·1 % and 61·4 % respectively and is considerably higher than for all the other pairs, where the correlation ranges between 30·9 % and 37·6 %. Correlation of the residues in different secondary structural types also follows the same trend. When α-helical residues are compared, the correlation is 20·8 % and 85·7 % for the pairs Con A–SBA and Favin–LcL respectively, whereas for other pairs it is zero. The correlation of β-sheet residues in 57·3 % and 69·8 % in these two pairs respectively and less than 45 % in the other pairs. Correlation of β-turn residues also follows the same pattern, where the correlation is 65 % in Con A–SBA pair and 66·7 % in Favin–LcL pair and lies between 37 and 47 % in other pairs. These results suggest a closer relation between Con A and SBA, and between Favin and LcL than in the other pairs which can be taken to indicate a divergence in the evolution of these lectins.

Comparison of the predicted β-turn sites is depicted schematically in figure 2. When the β-turn sites were scanned to see correlating sites within ± 2 residues for each turn, in all the pairs, it was observed that there is a high correlation between the sites in Con A and those in SBA and between the sites in Favin and LcL. When the predicted β-turn sites of Con A were compared with those obtained from X-ray crystallographic investigations, a high degree of correlation (77 %) was observed. Earlier, a correlation analysis of the occurrence of β-turns for proinsulin-c-peptides, pancreatic ribonucleases and proteinase inhibitors by Chou and Fasman (1979b), showed that these chain ancestor, we have carried out a correlation analysis of the β-turn regions of these proteins, and the details of such an analysis are given in table 5. When a cutoff value of $0{\cdot}75 \times 10^{-4}$ was applied for the β-turn propensity to identify the probable bend regions in these proteins, and the percentage correlation calculated, the highest correlation values were associated with the two pairs Con A–SBA and Favin–LcL, while the remaining pairs showed considerably less correlation.

The β-turn propensity of any tetrapeptide, W-X-Y-Z is obtained by multiplying the propensities of W, X, Y and Z to be in positions 1, 2, 3 and 4 respectively, of a β-turn. If, a residue in this tetrapeptide, say X, is replaced during evolution, by another residue with a lower propensity to be in the 2nd position of a β-turn, the overall turn propensity of the tetrapeptide will decrease. Such a replacement is more likely to bring down the turn propensity below the cutoff value in a tetrapeptide with low turn propensity, than in one with a high turn propensity. Therefore, the predicted sites with higher turn propensity are more likely to be conserved during the course of evolution than those with lower bend propensity. Therefore, if the cutoff value for identifying the β-turn regions is increased, the percentage correlation between two conserved proteins should increase; however, for non conserved proteins, where the correlation is due to random matching, this would result in a decreased correlation because the number of sites would be decreased. Hence we reasoned that if the higher correlation in the Con A–SBA and Favin–LcL pairs is due to a closer evolutionary relationship between the constituents of each pair, the percentage correlation should continue to remain high, and for other pairs it should decrease, which has indeed been observed. The correlation values for Con A–SBA and Favin–LcL pairs, remain in the same range when the cutoff values were increased to $1{\cdot}25 \times 10^{-4}$ and $2{\cdot}0 \times 10^{-4}$, whereas for other combinations it decreases dramatically, further confirming that the evolutionary relations between Con A and SBA and between Favin and LcL are much closer than in the other combinations.

In summary, these results indicate that the 4 legume lectins fall into two classes, Con A and SBA forming one Favin and LcL forming the other. This bifurcation points to a divergence in the course of their evolution from a common ancestor. Since there are only a limited number of legume lectin sequences available at present, it is difficult to predict whether this divergence has branched into more than two classes, which could be better understood when the primary structures of more legume lectins are known.

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Conditional rifampicin sensitivity of a *rif* mutant of *Escherichia coli*: rifampicin induced changes in transcription specificity

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Abstract. A *rif* mutant of *Escherichia coli* that exhibits medium and temperature-dependent sensitivity to rifampicin is described. In the absence of rifampicin, this strain grows in minimal and rich media at 30°C and 42°C. In its presence it is viable in rich medium at both temperatures, but in minimal medium only at 30°C. In minimal-rifampicin medium at the higher temperature, RNA synthesis is decreased. The addition of certain divalent salts ($MgSO_4$, $CaCl_2$, $BaCl_2$) in excess, or chelators (EDTA, EGTA, *o*-phenanthrolein) greatly increase viability in minimal-rifampicin medium at 42°C. Excess $MgSO_4$ (10 mM) also increases the rate of RNA synthesis in the same medium. A model is proposed wherein the *rif* mutation is suggested to cause a structural change in RNA polymerase that allows the binding of rifampicin and other ligands at 42°C. Rifampicin-binding is suggested to alter the conformation of RNA polymerase, impairing its ability to express genes required for growth in minimal medium. Implicit in this view is the assumption that these genes are structurally different from those expressed in rich medium in respect of certain template features recognized by RNA polymerase.

Keywords. Transcription; transcription specificity; rifampicin.

Introduction

Earlier reports from our laboratory described the isolation, genetic mapping and physiological characterisation of a temperature-sensitive transcription mutant of *Escherichia coli* in which the mutation mapped at a locus different from the loci coding for the subunits of RNA polymerase (Jabbar and Jayaraman, 1976, 1978; Jabbar, 1979; Jayaraman and Jabbar, 1980). It was suggested that the product of the locus could be an accessory transcription factor. In an attempt to obtain genetic evidence in support of this notion Dass and Jayaraman (1985) isolated a rifampicin-resistant (*rif*), temperature-insensitive derivative from a mutant which originally harboured the temperature-sensitive mutation. Genetic analysis of this derivative (Dass and Jayaraman, 1985) showed (i) the *rif* mutation was not responsible for the temperature-insensitive phenotype of this derivative and (ii) a second mutation mapping in the same *rif* mutation present in the temperature-insensitive derivative. The results suggest that this mutation leads to changes in transcription specificity under certain conditions.

Materials and methods

Media and chemicals

MA minimal medium and LB nutrient medium were used for all experiments (Miller, 1972). Additional salts were used at the indicated concentrations. Chelators were added at 0·1 mM, and rifampicin at 100 μg/ml. Rifampicin and *o*-phenanthrolein were from Sigma Chemical Co., St. Louis, Missouri, USA, while all other chemicals were from local sources. [^{3}H]-Uridine was from Bhabha Atomic Research Centre, Bombay.

Bacterial strains

The bacterial strains used in this study are listed in table 1.

Table 1. List of *E. coli* strains used in this work.

Strain	Relevant genotype	Source/construction
KL16	Hfr *rel*A	B. J. Bachmann
GMS343	F$^-$ *aro*D *arg*E *rps*L	B. J. Bachmann
CSH57	F$^-$ *his trp pur*E *leu met*A *ilv*D *rps*L *arg*G	Cold Spring Harbor collection
BJ240	F$^-$ *his trp pur*E *leu met*A *arg*G *rps*L *fit*76-*fit*24 *rif* (*rpo*B240)	Derivative of CSH57; Dass and Jayaraman (1985)
BJ5701	F$^-$ *his trp pur*E *leu met*A *rps*L *arg*G *rif* (*rpo*B)	Spontaneous Rif^r mutant of CSH57
BJ5702	F$^-$ *his trp pur*E *leu rps*L *arg*G *rpo*B240	Met$^+$ Rif^r derivative of CSH57 obtained from the cross P1/BJ240 × CSH57

Methods

P1 transductions were done as described by Miller (1972). For studying RNA synthesis, cells were grown to mid-log phase at 42°C in minimal medium, with or without rifampicin. Cultures were adjusted to the same turbidity and labelled with [^{3}H]-uridine (12·7 Ci/mmol; 1 μCi/ml). At intervals, during a period of 30 min, samples were treated with ice-cold 10% trichloroacetic acid containing 100 μg/ml uridine, filtered on membrane filters, washed and counted in a Beckman Liquid Scintillation Counter. Incorporation was linear with time.

Results

*Mapping of the rif mutation in BJ*240

This paper describes the properties of the *rif* mutation present in the strain BJ240 described earlier (Dass and Jayaraman, 1985). First of all, it was necessary to ensure that this mutation maps at the *rpo*B locus (90 min; Bachmann, 1983) as almost all *rif* mutations do (Yura and Ishihama, 1979). Data presented in table 2 show that the *rif* mutation in BJ240 is cotransducible with *arg*E or *met*A at approximately the same frequency (50%) which would be expected of mutations in the *rpo*B locus. It can therefore be concluded that the *rif* mutation under study lies in *rpo*B. Accordingly it was designated as *rpo*B240.

Table 2. Transductional mapping of the *rif* mutation in BJ240.

Cross	Donor	Recipient	Selected marker	Unselected marker	Cotransduction frequency (%)
1	BJ240 (*rif*)	GMS343 (*arg*E)	*arg*E$^+$	*rif*	49 (78/159)
2	KL16 (*rif*$^+$)	BJ240 (*met*A *rif*)	*met*$^+$	*rif*$^+$	47 (435/918)

rif—Resistance to rifampicin.
rif$^+$—Sensitivity to rifampicin.

*Viability of BJ*5702

The *rpo*B240 mutation was transduced into an *rpo*B$^+$ strain, CSH57. The resultant transductant, BJ5702, was used for the studies described below. Viability of BJ5702 under different conditions was compared with that of its parent (CSH57) and BJ5701 (a spontaneous *rif* mutant of CSH57). It can be seen from table 3 that in the absence of rifampicin, BJ5702 behaves just like CSH57 and BJ5701. In LB-rifampicin medium, BJ5702 was fully viable at 30°C and 42°C. In minimal-rifampicin medium, however,

Table 3. Viable colony counts of the strains in the presence and absence of rifampicin at 30°C and 42°C in LB and minimal media.

	Minimal medium				LB medium			
	– Rifampicin		+ Rifampicin		– Rifampicin		+ Rifampicin	
Strain	30°C	42°C	30°C	42°C	30°C	42°C	30°C	42°C
CSH57 (*rpo*B$^+$)	100	100	10^{-7}	10^{-7}	105	108	10^{-6}	10^{-6}
BJ5701 (*rif*)	100	97	94	103	103	99	101	100
BJ5702 (*rpo*B240)	100	101	99	5×10^{-2}	99	98	107	86

drastically. The few colonies that appeared at the higher temperature (at a frequency of approximately 5×10^{-4}) continued to be similarly non-viable on subsequent plating on minimal-rifampicin plates at 42°C (data not shown). There is thus a medium and temperature-dependent switch in the phenotype of BJ5702 from rifampicin resistance to sensitivity.

*RNA synthesis in BJ*5702

Is the non-viability of BJ5702 under the above conditions a reflection of the sensitivity of transcription to rifampicin? When a culture of the mutant was inoculated in minimal-rifampicin medium at 42°C to an initial density of about 10^7 cells/ml (as is usually done), the culture grew as well as it did in the absence of rifampicin (data not shown). This was in seeming contradiction to the results presented above. In liquid medium, under the usual culture conditions, cells undergo not more than 9 divisions before cessation of growth. On the other hand, a colony on a plate arises from a single cell and is the result of at least 24 cell divisions. It was therefore decided to increase the duration of growth of BJ5702 in liquid medium such as to mimic growth conditions on plates. The strain was subcultured in rifampicin-minimal medium at 42°C to an initial cell density of 5×10^2 cells/ml. The culture did grow, but at a decreased rate and ceased growth at a smaller cell density, relative to the control (no rifampicin; data not shown). It is not clear how cells that are largely non-viable on plates can grow in liquid medium. A possible explanation is that initially cells grow normally at 42°C. With increasing time of exposure to rifampicin they slacken in their growth rate and ultimately cease to grow altogether, but nonetheless, allowing a small random population to escape the inhibitory action of the antibiotic.

[^{3}H]-Uridine incorporation was studied in cultures grown at 42°C from an initial density of 10^7 cells/ml. There was no difference in incorporation in the presence or absence of rifampicin (table 4). Thus under the usual experimental conditions, RNA synthesis in BJ5702 is resistant to inhibition by rifampicin. This result (coupled with the fact that BJ5702 is viable on LB-rifampicin plates) almost certainly demonstrates that RNA polymerase in this strain is a rifampicin-resistant enzyme. [Under the same conditions, the *rpo*B$^+$ enzyme of the wild-type strain CSH57 is sensitive to rifampicin (table 4)]. BJ5702 was next grown from an initial density of about 5×10^2 cells/ml. In the culture grown in the presence of rifampicin, incorporation was considerably less (table 4). An implicit assumption in correlating [^{3}H]-uridine incorporation and

Table 4. Incorporation of [^{3}H]-uridine at 42°C in cultures grown from different initial densities in the presence and absence of rifampicin.

Strain	Initial cell density (cells/ml)	Incorporation/30 min (cpm)	
		– Rifampicin	+ Rifampicin
CSH57 (*rpo*B$^+$)	10^7	3223	105
BJ5702 (*rpo*B240)	10^7	2552	2640

viability is that incorporation represents all the species of RNA needed for growth under a given set of conditions. This need not necessarily be true with BJ5702 labelled at 42°C in the presence of rifampicin. There could be qualitative differences between RNA synthesized in minimal medium at 42°C in the presence and absence of the antibiotic. Such differences will not show up markedly in labelling experiments, but could be seen as loss of viability. Colony forming ability is therefore a more reliable index of rifampicin-sensitivity/resistance than the rate of [^{3}H]-uridine incorporation.

Effect of cations on rifampicin-sensitivity

Is BJ5702 'conditionally auxotrophic' in minimal-rifampicin medium at 42°C? Addition of various amino acids in different combinations failed to reverse the phenotype of this strain (data not shown). We also wondered if changes in the composition or concentration of media components could reverse its phenotype to rifampicin-resistance. During the course of experiments designed to test this view, it was observed that the addition of excess $MgSO_4$ to minimal-rifampicin medium increased the viability of BJ5702. This observation was followed up by studying the effect of a number of divalent cations. These were: Zn^{2+} (an innate constituent of RNA polymerase); Mg^{2+} (essential for the activity of RNA polymerase); Mn^{2+} (which can substitute for Mg^{2+} in certain transcription reactions); Ca^{2+} (a physiologically important ion) and Ba^{2+} (a randomly chosen ion). Salts of these divalent cations were added to minimal-rifampicin medium at 42°C to test their effect on the viability of BJ5702. It is apparent from table 5 that while $ZnSO_4$ had no effect and $MnCl_2$ a partial effect, $MgSO_4$, $BaCl_2$ and $CaCl_2$ greatly reversed the rifampicin-sensitivity of this strain.

Table 5. Effect of divalent salts on the viability of BJ5702 in minimal medium at 42°C with and without rifampicin.

	−Salt		+Salt (0·1 mM)		+Salt (1 mM)		+Salt (10 mM)	
Salt	−Rif	+Rif	−Rif	+Rif	−Rif	+Rif	−Rif	+Rif
$ZnSO_4$	100	4×10^{-2}	N.D	N.D	103	7×10^{-2}	99	4×10^{-2}
$MgSO_4$	100	5×10^{-2}	N.D	N.D	97	4×10^{-2}	101	35
$MnCl_2$	100	8×10^{-2}	N.D	N.D	98	3×10^{-2}	98	3
$CaCl_2$	100	4×10^{-2}	102	3×10^{-2}	105	32	N.D	N.D
$BaCl_2$	100	2×10^{-2}	N.D	N.D	100	5×10^{-2}	105	83

Colony counts normalized to the number (taken as 100) on plates without added salt and rifampicin.
N.D.—Not determined.

One possibility was that excess of salts/ions in the medium limited the effective concentration of rifampicin per cell, perhaps by complexing with the antibiotic, or by the viability of CSH57 was inhibited by rifampicin to the same extent irrespective of the addition of 10 mM $MgSO_4$ (data not shown).

The possible effect of excess $MgSO_4$ on RNA synthesis in BJ5702 was studied. Cells were grown at 42°C, starting from an initial density of about 5×10^2 cells/ml. The culture grown in the presence of rifampicin and 10 mM $MgSO_4$ incorporated more [^{3}H]-uridine (1·5 fold) than the culture grown in rifampicin, but without excess $MgSO_4$. Thus the ability of BJ5702 to grow on rifampicin-plates in the presence of $MgSO_4$ can be accounted for, at least in part, by a positive effect of Mg^{2+} on transcription. In line with the conclusions drawn earlier, Mg^{2+} (and possibly other divalent cations) perhaps delays or prevents an eventual transition of cells to rifampicin-sensitivity in minimal medium at 42°C, allowing for a greater fraction to undergo sufficient divisions and form colonies.

*Influence of chelators on viability of BJ*5702

If divalent cations can reverse the phenotype of BJ5702 to rifampicin-resistance, would the addition of ion-chelators increase the sensitivity of this strain? Three commonly used chelators were chosen, namely, EDTA, EGTA and *o*-phenanthrolein. These were added to minimal-rifampicin medium at a non-lethal concentration of 0·1 mM. All the 3 chelators greatly restored the viability of BJ5702 in minimal-rifampicin medium at 42°C (table 6).

Table 6. Influence of chelators on the viability of BJ5702 in minimal-rifampicin medium at 42°C.

−Chelator			+EDTA		+EGTA		+*o*-phenanthrolein	
−Rif	+Rif	+Rif+ $MgSO_4$ (10 mM)	−Rif	+Rif	−Rif	+Rif	−Rif	+Rif
100	3×10^{-2}	51	96	29	98	32	95	23

Normalized to the number (taken as 100) on plates without added chelator and rifampicin. Chelators were added to a final concentration of 0·1 mM.

Discussion

We have described in this paper some properties of a strain harbouring a particular *rif* mutation, *rpo*B240. This mutant is conditionally sensitive to rifampicin, depending on the medium, temperature, and the presence/absence of (excess) divalent cations, or metal chelators. Such behaviour can be partially correlated with the pattern of RNA synthesis. Our results can be rationalized by proposing specific conformational changes in the *rpo*B240 RNA polymerase as a result of temperature, and the binding rifampicin, ions, or chelators to the enzyme.

(Ovchinnikov *et al.*, 1983). Conformational changes in the enzyme attendant on rifampicin binding have been proposed to be of importance in its activity (Krakow *et al.*, 1976; Nakamura and Yura, 1976; Doi, 1977; Reisbig *et al.*, 1982). Current evidence suggests that rifampicin may bind to RNA polymerase from *rif* mutants also. Some of these mutants are sensitive to derivatives of the anitibiotic. These derivatives act on the *rif* polymerase by a mechanism similar to that of rifampicin on the wild-type enzyme, possibly through interaction with the same enzyme site (Nikiforov *et al.*, 1982). Metal ions like Mg^{2+} and Mn^{2+} bind to the substrate-binding site (Goldthwait *et al.*, 1970) located in the β-subunit (Armstrong *et al.*, 1976) and bring about structural changes in the enzyme (Krakow *et al.*, 1976). A Co^{2+}-containing RNA polymerase shows alterations in promotor recognition properties with T7 and λ *plac*5 templates (Speckhard *et al.*, 1977). Various lines of evidence, including enzyme reconstitution studies, suggest that Zn^{2+} in RNA polymerase could be involved in maintaining a required conformation (Krakow *et al.*, 1976; Zillig *et al.*, 1976). Chelators like *o*-phenanthrolein and picolinic acid are believed to inhibit transcription (specifically at the initiation step) by forming a ternary complex with zinc in RNA polymerase (Scrutton *et al.*, 1971; Speckhard *et al.*, 1977; Collins *et al.*, 1979). During a period of transient growth inhibition by chelators, it has been proposed that the proteins synthesized are quantitatively and qualitatively different from those made during normal growth (Collins *et al.*, 1979).

We propose a model that may be quite consistent with the above reports. The *rpo*B240 mutation is suggested to cause a change in RNA polymerase such that it is conformationally altered, but not functionally inactivated, at the higher temperature. Such altered topology allows the binding of rifampicin and other ligands like metal ions and chelators to the enzyme. The rifampicin-bound polymerase is affected in transcription specificity such that it fails to express at 42°C the genes required for growth in minimal medium. Metal ions and chelators possibly revert the conformation of the polymerase–rifampicin complex to one that can express these genes.

An implicit assumption of this model is that genes expressed in minimal medium and those expressed in rich medium are not only functionally different (anabolic *versus* catabolic), but also structurally so. The selective transcription of genes has been partly attributed to the extent of supercoiling of different regions of the chromosome (Smith, 1981). The number of such supertwisted template domains and their degree of coiling may depend on, among other things, the medium of growth (Worcel and Burgi, 1972; Sinden and Pettijohn, 1981). The amino acid biosynthetic operons have characteristic structural features that ensure optimal expression in minimal medium and attenuation in rich medium (Kolter and Yanofski, 1982). Several catabolite sensitive operons have a special promotor sequence recognized by the CAMP-CAP complex (Rosenberg and Court, 1979). A number of operons stringently regulated under amino acid starvation have a sequence called the 'discriminator' that sets them apart from other genes (Travers *et al.*, 1981).

It is possible that the 'minimal medium genes' differ from the 'LB medium genes' in respect of certain template features (sequences, degree of coiling etc.) recognized by result of inability to express one or more genes required for the biosynthesis of any one amino acid have been reported (Morishita and Yura, 1976; Ryu and Takayanagi, 1979). However, this is not likely to be the case with BJ5702 since the addition of different amino acids to minimal-rifampicin medium at 42°C did not restore colony forming ability of this strain (see above).

Our model proposing that rifampicin can alter transcription specificity derives further support from experiments reported by many others. Rifampicin may cause activation of promoters and read-through past terminators (Newman *et al.*, 1982; Fukuda and Fujimori, 1983), and affect varied cellular processes (Buchstein and Hinkle, 1982; Mirkin and Shmerling, 1982; Ito and Ohnishi, 1983; Polaczek and Ciesla, 1983). In addition, we have observed that *rpo*B240 mutation, in combination with different *fit* mutations, leads to changes in the expression of a number of gene groups, including phage genomes and the lactose operon, and also alters the viability of strains in minimal medium. These results will be published elsewhere.

Acknowledgements

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Failure of exogenous methylated adenines to induce cytotoxic and genotoxic effects in yeast auxotrophic to adenine

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Abstract. Alkylation of DNA is an important step in the biological effects of alkylating agents. In an attempt to determine the effect of alkylation at N 1 and N 3-positions in adenine, diploid yeast auxotrophic to adenine was cultured in synthetic medium in which adenine was replaced by N 1 or N 3-methyladenines. The expectation was that, the cells will incorporate methyladenines to the DNA newly synthesised by the salvage pathway and thus facilitate the expression of their biological effects, if any. The biological end points monitored were cell killing, gene conversion and reverse mutation. Contrary to expectation, no growth occurred in the cultures even after 48 h of incubation. The cells retained viability, but were only arrested in growth. When subcultured in the presence of sub-optimum levels of both adenine and N 1-methyladenine complete growth occurred. However, no enhancement in the induction of gene conversion or back mutation was observed in these cultures.

Experiments with tritium labelled N 1-methyladenine showed that it is taken up by the cells. Hence, it is speculated that the ineffectiveness of methyladenines is due to their not being utilised as substrates in the synthesis of DNA by the salvage pathway. This is important for the assessment of the biological effects of DNA precursor pool alkylation.

Keywords. Methylated adenines; yeast; biological effects.

Introduction

The importance of DNA alkylation in the mutagenic and carcinogenic action of alkylating agents is well recognised (Lawly, 1976). DNA is a complex molecule. It contains several sites with different nucleophilic strength for reaction with alkylating agents. Figure 1 shows the alkylable sites in DNA along with sites for hydrogen bonding between complementary bases. The biological effects of alkylation are known only for a few of these sites. Loveless (1969) proposed that guanine alkylated at 06 position may mispair with thymine during DNA replication leading to AT-GC transition type mutagenesis. Subsequently a number of workers have experimentally demonstrated the specificity of 06-guanine alkylation for inducing forward mutations in cultured Chinese hamster cells (Newbold *et al.*, 1980; Suter *et al.*, 1980; Beranek *et al.*, 1983).

Alkylation of many other sites shown in figure 1 can also result in their mispairing during DNA replication and thus can be potentially mutagenic (Singer, 1979; Lawley,

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Abbreviations used: YEPD: Yeast extract, peptone, dextrose; SM, synthetic medium

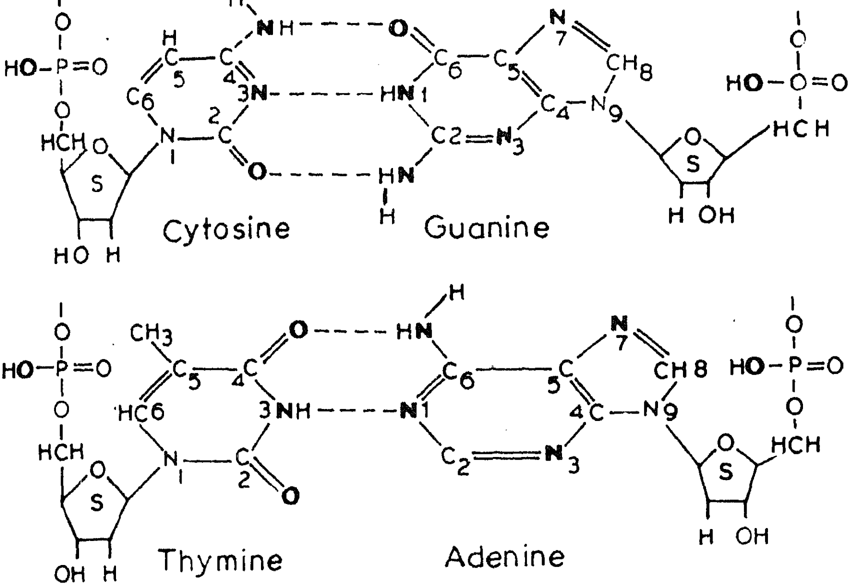

Figure 1. Hydrogen bonding and alkylable sites (in bold letters) in DNA.

1976). However, the biological effects of alkylation at many of these sites have not been established, probably because of the very low level of *in vivo* alkylation. Further, their low effectiveness, if any, may be masked by alkylation at more effective sites (such as 06 guanine). One of the approaches to study the effect of alkylation at specific sites is to grow cells in a medium supplemented with specific alkylated bases, under conditions where the cells are forced to utilise them. Under such conditions it is expected that the alkylated bases get incorporated into the freshly synthesised DNA and exhibit their biological effects, if any. Such an attempt has been reported for Chinese hamster V79 cells in which the *de novo* purine synthesis was biochemically inhibited (Ball *et al.*, 1983). Alternatively adenine requiring yeast strains can be used as a more natural system for such investigations. In these systems *de novo* purine synthesis is interrupted by a mutation in one of the adenine loci. The cells grow only in the presence of exogenous adenine which is utilised to synthesise nucleic acids through the salvage pathway.

In this paper, we report on the effect of 1 and 3-methyladenines on the growth, and induction of genetic effects in an adenine requiring strain of yeast *Saccharomyces cerevisiae*. These results show that, contrary to expectation, both the alkyladenines fail to induce cytotoxic and genotoxic effects in this strain.

Materials and methods

Diploid yeast *S. cerevisiae* BZ34 was used in these studies. The genotype of the strain is shown in figure 2. It is homozygous for mutation at *ade 2* locus which inhibits synthesis

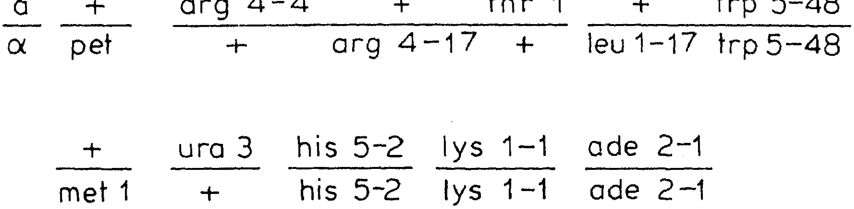

Figure 2. Genotype of diploid yeast BZ34. The strain is auxotrophic to the base adenine and a number of amino acids including arginine and histidine.

yeast extract, peptone, dextrose (YEPD) medium or when adenine is supplemented in the synthetic medium (SM). The strain also carries a number of other mutations, the important ones from the point of view of the present study being (i) the non-complementing mutant alleles at the argininosuccinase locus and (ii) homozygous mutations at the histidine locus. On account of these mutations, the strain also requires arginine and histidine to be supplemented in the growth medium. As genetic end points in the present study, prototrophs to arginine and histidine were monitored. These arise as a result of two independent genetic events-gene conversion (non-reciprocal recombination) and back mutation respectively. Experiments in our laboratory have shown that alkylating agents such as ethylmethane sulphonate (Murthy and Shankaranarayanan, 1978), methylmethane sulphonate (Anjaria and Madhvanath, 1983), N-methyl-N′-nitro-N-nitrosoguanidine (Murthy, M. S. S. and Deorukhakar, V. V., unpublished results) induce, in addition to cell killing, gene conversion and histidine revertants in this strain in a dose-dependent manner. These effects are attributed to the alkylation of DNA. The genetic end points can be easily detected by plating treated cells on SC medium plates lacking in that particular amino acid. Culture growth conditions and composition of various media have been described earlier (Murthy *et al.*, 1975). Cells are routinely subcultured in YEPD medium at 30°C for 48 h at which time they reach stationary phase.

N1-Methyladenine was purchased from Sigma Chemical Co., St. Louis, Missouri, USA. N3-Methyladenine was a generous gift from Dr. B. Kaina. Part of N1-methyladenine was radioactively labelled by catalytic exchange reaction with tritium gas. All other chemicals were of analar grade purchased locally.

Treatment of cells with methylated bases

Cells from stationary phase culture were washed and resuspended at a titer of 10^5 cells/ml. Out of this, 0·2 ml was inoculated into a culture flask containing 20 ml SC medium in which adenine was replaced by N1-methyladenine or N3-methyladenine (20 mg/L) and incubated in a shaker water bath at 30°C. A concentration of 20 mg/L was chosen because this is the optimum concentration of adenine required for growth. A control culture in which the medium was supplemented with unsubstituted adenine was also maintained under similar conditions. In a second set of experiments, culture flasks growth had occurred, cell number was also counted using a hemocytometer. For determining the frequency of induction of genetic end points, appropriate cell titers (10^5/ml or more) were plated on 4 omission plates (lacking arginine or histidine as the case may be). The plates were stored in dark at 30°C and colony counts were made after 3 to 5 days.

In some experiments, at the end of 24 h incubation, cultures containing methylated adenine were divided into two parts. In one part, methylated adenine (N1 or N3 as the case may be) was replenished to a level of 20 mg/L. To the other part adenine was added to a final concentration of 20 mg/L and the incubation continued for another 24 h. Alternatively, subculturing was done in the presence of sub-optimal concentration of adenine (5 and 10 mg/L) alongwith 5 mg/L of N1-methyladenine. Experiments were repeated at least 2 to 3 times under each condition.

Results and discussion

It has been suggested that alkylation at N3 position in adenine could result in its mispairing with cytosine during DNA replication (Lawley, 1976; Nagata *et al.*, 1963). The coding property of N1 alkyladenine is not known. N1 position participates in hydrogen bonding with its complementary base (figure 1). Adenine alkylated at this position is reported to behave ambiguously in transcription experiments (Kroger and Singer, 1979).

Culture growth under N1-methyladenine supplementation, as monitored by hemocytometer counts and colony counts are shown in table 1. It can be seen that while the adenine supplemented cultures grow to stationary phase over 48 h period no growth

Table 1. Growth kinetics of diploid yeast auxotrophic to adenine, in synthetic medium supplemented with either adenine or N1-methyladenine. Values are mean of two experiments. Similar results were obtained with N3-methyladenine supplementation.

Base supplemented	Period of incubation		
	0 h	24 h	48 h
	Number of cells per ml		
Adenine (20 mg/L)	100	$3{\cdot}8 \times 10^5$	$1{\cdot}5 \times 10^7$
	1×10^6	$1{\cdot}4 \times 10^7$	—
N1-methyladenine (20 mg/L)	100	104	114
		104	$1{\cdot}3 \times 10^5$* ($1{\cdot}6 \times 10^7$**)
	$1{\cdot}0 \times 10^6$	$0{\cdot}9 \times 10^6$	—

* Adenine (20 mg/L) supplemented after 24 h of incubation in the presence of N1-methyladenine. No growth occurred in the culture in which N1-methyladenine was replenished.

methyladenine. This was so, for both low and high starting cell titers. The data of low cell titer (100 cells/ml) are based on colony counts. At high cell titer the colony counts agreed with the hemocytometer count within the plating efficiency of the cultures (80–100%). This suggests that the arrested cells were completely viable. This was further confirmed in the experiments in which 1-methyladenine treated cells were supplemented with normal level of adenine after 24 h of incubation. Such cultures grew to stationary phase within the next 48 h. Microscopic examination of high starting titer samples showed that the arrested cultures had 50–60% of cells with buds at both 24 and 48 h.

Cultures in which sub-optimum levels of adenine (5 and 10 mg/L) were supplemented simultaneously with N1-methyladenine (5 mg/L) grew to the same extent as control cultures with only adenine. Data on the induction of gene conversion and reverse mutation for these cultures are shown in table 2. It is seen that the frequency of induction of these end points did not increase in these cultures when compared with the spontaneous frequency in cultures growing in adenine supplementation.

Table 2. Non-induction of cell killing and genetic end points in diploid yeast grown in synthetic medium containing a mixture of adenine and N1-methyladenine. The initial cell titer was 10^3/ml.

	After 24 h of incubation		
Supplemented purine base	Cell number	Gene conversion per 10^6 survivors	Histidine revertants per 10^7 survivors
Adenine (10 mg/ml)	$1{\cdot}99 \times 10^6$	80	26
5 mg adenine + 5 mg N1-methyladenine	$1{\cdot}85 \times 10^6$	86	21
10 mg adenine + 5 mg N1-methyladenine	1×10^6	85	25

The absence of growth, cytotoxic and genotoxic effects in cultures supplemented with methyladenines observed in this study may be interpreted to mean that (i) either the alkyladenines are not entering the cell or (ii) they are not being utilised in the synthesis of nucleic acids by the enzymes of the salvage pathway. However, the yeast strain used in this study is adenine dependent and hence must have an effective mechanism to transport it across the cell membrane. This is supported by the growth data given in table 1 under adenine supplementation. Further more, yeast cells are also known to be permeable to a number of methylated xanthines (Vashishat and Kaushal, 1983).

In order to further investigate the transport of alkyladenines across cell membrane, was spiked with a 10 μCi of high specific activity [H]-N1-methyladenine. After 4 h of incubation, 5 ml suspension was centrifuged (for 10 min at 5000 RPM), supernatant removed and washed in 5 ml water. The washing was repeated four more times. At the end of the 5th washing, the cells were resuspended in 5 ml water. 0·2 ml of supernatant from each wash and 0·5 ml of the final cell suspension were taken into vials containing 10 ml scintillation cocktail (Toluene-400 ml; POPOP-40 mg; PPO-2 gm; Triton X-100–200 ml) and counted for tritium radioactivity in a Packard Tricrab 4530 liquid scintillation counter. The radioactivity data are shown in table 3. It is seen that radioactivity in the supernatant rapidly decreased after each wash and reached just about twice the background level after the 5th wash; on the other hand the activity in the cell suspension after the 5th wash was about 35 times higher than the background activity suggesting that [^{3}H]-N1-methyladenine had indeed penetrated the cell membrane. Under the present circumstances, the absence of growth also means that methyladenines are not being demethylated inside the cells.

Table 3. Uptake of N1-methyladenine by cells

Number of washings	Radioactivity in wash/pellet (counts per min)
0	$3{\cdot}27 \times 10^5$
1	$2{\cdot}5 \times 10^4$
2	$1{\cdot}7 \times 10^3$
3	$1{\cdot}19 \times 10^2$
4	65
5	50
Cell suspension	858

0·2 ml of supernatant from each wash was counted. After all the washings, the pellet was suspended in 5 ml water of which 0·5 ml was used for measuring radioactivity.

Alternatively it may be possible that even after entry into the cells, the methylated adenines may not be utilised for DNA synthesis, suggesting that they are not suitable substrates for the enzymes involved in the salvage pathway. Ball *et al.* (1983), Kaina and Aurich (1984) have shown that 06-methylguanosine and 06-methylguanine supplied exogenously were not incorporated into DNA of V79 Chinese hamster cells. Thus, eventhough we were unable to induce any biological effects by incubating cells in the presence of specific methyl adducts of adenine, the present study, along with others suggests that the inability to incorporate alkylated bases (and nucleosides) may be a general cellular phenomenon. The cells appear to have a machinery to exclude from its biosynthetic pathways, methylated purine base which, if incorporate into DNA may lead to cytotoxic and genotoxic effects. If so, this has significance in the assessment of the biological effects of alkylation of DNA precursor pool.

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Short Communication

Variable association of DNA polymerase with the cytoskeletal fractions of resting and dividing cells

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Abstract. A DNA polymerase activity associated with the detergent insoluble cytoskeletal fraction has been identified in dividing and non-dividing rat hepatocytes and a hepatoma (the Zajdela Ascitic Hepatoma). About 35 % of the enzyme is found associated with the cytoskeletal fraction of non-dividing cells as compared to about 3–6 % of the enzyme in dividing cells even though the dividing cells contain larger amounts of the extranuclear enzyme. The properties of the enzyme are similar to those of DNA polymerase-α. It is suggested that the association of the enzyme with the cytoskeletal fraction has functional significance.

Keywords. α-DNA polymerase; cytoskeleton; Zajdela Ascitic Hepatoma.

Introduction

The intracellular location of DNA polymerase-α, the major DNA replicating enzyme has been controversial (Sarngadharan *et al.*, 1978). Recent immunocytochemical studies have indicated a predominantly nuclear location of the enzyme (Bensch *et al.*, 1982; Matsukage *et al.*, 1983; Nakamura *et al.*, 1984). But biochemical extraction procedures (Bollum and Potter, 1958; Keir, 1965) as well as certain immunofluorescence studies have indicated a large cytoplasmic pool (Brown *et al.*, 1981). There are, in addition, other reports indicating the association of the enzyme with cytoplasmic membrane (Baril *et al.*, 1970, 1971; Davis *et al.*, 1971). We have also reported a DNA polymerase-α like activity in the plasma membranes of a rat hepatoma, closely associated with the detergent insoluble fraction (Shashikant *et al.*, 1983).

A major discrepancy in presenting an exclusively nuclear location of the enzyme (Bensch *et al.*, 1982; Matsukage *et al.*, 1983; Nakamura *et al.*, 1984) arises from the fact that the site of synthesis of the enzyme is expected to be the cytoplasm. It is therefore surprising not to find the enzyme in the cytoplasm by immunocytochemical methods. We have now adopted a modified approach to address this question. Treatment of cells with a nonionic detergent solubilizes most of the cellular proteins (soluble fraction) leaving behind an insoluble material consisting of filamentous structure along with the nuclei. Intact nuclei can then be separated by double detergent treatment and low speed centrifugation. This supernatant is the cytoskeletal fraction (CSF) (Cervera *et al.*, 1981).

We have examined the distribution of DNA polymerase-α in these fractions from resting and dividing rat liver cells and a rat hepatoma (Zajdela Ascitic Hepatoma, ZAH) cells.

Materials and methods

Chemicals

Radioactively labelled nucleoside triphosphates were obtained from Amersham, England, cold triphosphates and synthetic templates from PL Biochemicals. All other chemicals were from Sigma Chemical Company, St. Louis, Missouri, USA.

Cell suspension

Livers were obtained from both normal and partially hepatectomised adult Wistar rats (30–36 h after hepatectomy). Fetal livers were obtained from 18–19 days old embryos. After perfusion with cold phosphate buffered saline (PBS) (135 mM NaCl, 2·75 mM KCl, 1·5 mM KH_2PO_4, 8 mM Na_2HPO_4, pH 7·2), the tissues were minced and gently homogenized in PBS (10 ml/gm of tissue) in a loose fitting homogenizer. After passing the homogenate through a nylon mesh (size 50–70 μ) it was centrifuged at 700 g to pellet the cells. The pellets were resuspended in 10 mM PIPES buffer, pH 6·8, containing 10 mM KCl, 1 mM phenyl methyl sulphonyl fluoride (PMSF) and 10% sucrose (w/v).

Tumour cells

ZAH cells were obtained from the ascitic fluid from tumour bearing Wistar rats maintained by serial transplantation in the laboratory (Shashikant *et al.*, 1983). The cells were harvested from the peritoneal cavity of the animals between the 5th and 7th day of transplantation, diluted immediately with PBS and pelleted down at 700 g and were washed twice with PBS.

Subcellular fractionation

Fractionations were performed according to the procedures already described (Cervera *et al.*, 1981). All operations were carried out at 4°C. Cells were resuspended at 2–4 $\times 10^6$/ml in PIPES buffer and NP40 was added slowly to make the detergent concentration 1% (v/v). The suspension was stirred for 15 min and then centrifuged at 1,000 g for 10 min. The supernatant (soluble fraction) was collected and preserved; the pellet was washed and resuspended in 1/3 of the original volume of buffer (10 mM Tris, pH 7·4, 10 mM NaCl, 1·5 mM $MgCl_2$, 5 mM $CaCl_2$ and 1 mM PMSF) and gently homogenised in a loose fitting homogenizer in the presence of 1% Tween 40 and 0·5% deoxycholate. The nuclei were pelleted by centrifugation at 750 g for 10 min. The

Assays of DNA polymerase (Shashikant *et al.*, 1983) 5′-nucleotidase (Gurd and Evans, 1974) alkaline phosphatase (Pekarthy *et al.*, 1972) succinic dehydrogenase (Earl and Korner, 1965) and pyrophosphatase (Shatton *et al.*, 1981) were carried out according to the methods described earlier.

Protein concentrations were determined by the Lowry method (Lowry *et al.*, 1951) in the presence of sodium dodecyl sulphate (SDS). Gel electrophoresis (Laemmli, 1970) and subsequent staining (Oakely *et al.*, 1980) were carried out as described elsewhere.

Results

Cytoskeletal fractions

Detergent treatment solubilizes about 70% of protein which includes marker enzymes such as pyrophosphatase, alkaline phosphatase and succinic dehydrogenase. However, 5′-nucleotidase, an enzyme found in association with plasma membrane skeleton of liver cells (data not shown), was retained on the CSF indicating a certain commonality between PM skeleton and CSF. Electrophoretic analysis further revealed (figure 1) many common proteins between PM skeleton (lane 2) and CSF (lane 1) of liver except for proteins P80, P59, P41 and P40 which are specific to CSF. A similar comparison of ZAH-CSF (lane 3) with ZAH-PM skeleton (lane 4) shows protein P120, P83, P77, P68 and P51 are specific to ZAH-CSF. However, PM skeleton of ZAH appears to have a distinct profile as compared to CSFs and PM skeleton of liver. A comparison of protein profiles of CSF fractions of liver and ZAH show distinct patterns with characteristic proteins in each cell type.

DNA polymerase activity in the soluble fraction and in CSF.

The DNA polymerase activities determined using activated calf thymus DNA as template from both soluble and CSF were inhibited by NEM. The percentage inhibition in the presence of 1 mM N-ethylmaleimide (NEM) varied between 82–87% in the different samples tested. None of the samples examined showed any activity with synthetic polymers such as poly rA:oligo dT or poly rC:oligo dG or with a ribopolymeric template, AMV RNA as templates (results not shown). These properties suggest that the enzyme is likely to be DNA polymerase-α and also rule out contamination of DNA polymerising activities such as γ-polymerase and reverse transcriptase in the activity that we are assaying.

Distribution of DNA polymerase activity

The amounts of DNA polymerase activity from the different samples examined are

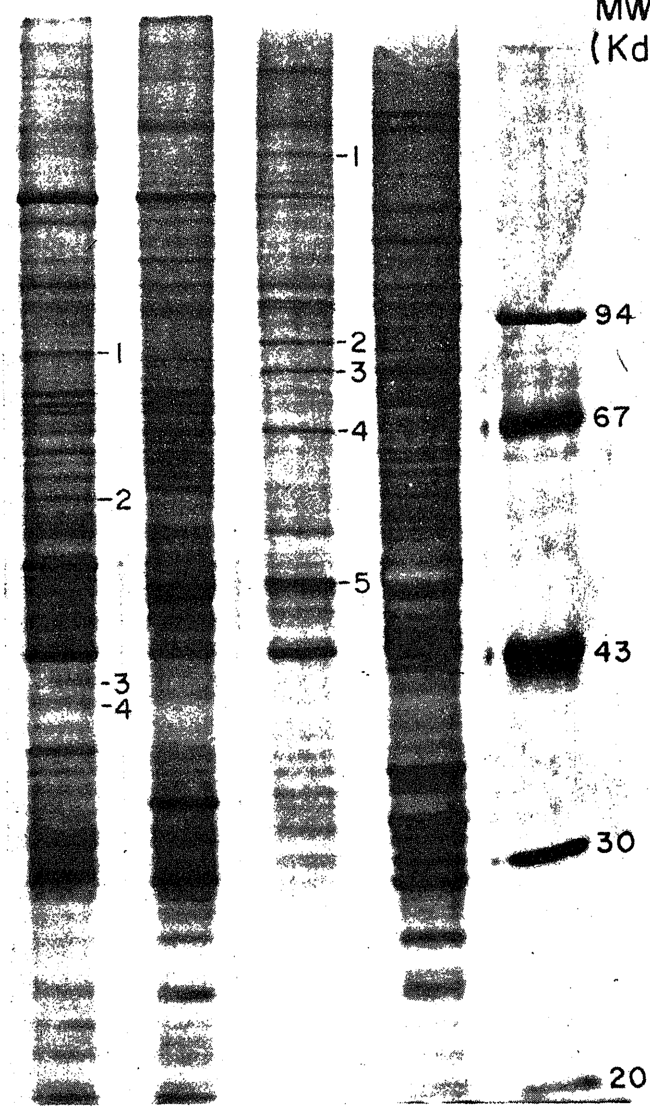

Figure 1. SDS polyacrylamide gel electrophoresis (10%) profile of proteins from plasma membrane skeletons and CSF isolated from liver and ZAH cells. Lane 1, liver CSF; Lane 2, liver PM skeleton; Lane 3, ZAH CSF; Lane 4, ZAH PM skeleton; Lane 5, molecular weight markers (obtained from Pharmacia). Protein bands: Lane 1, bands 1, 2, 3 and 4 are P80, P59, P41 and P40 respectively. Lane 3: bands 1, 2, 3, 4 and 5 are P120, P83, P77, P68 and P51 respectively.

associated with CSF in these cells is larger than that present in the corresponding fractions obtained from dividing cells. Table 1 also shows that there is considerable variation in the amount of enzyme present per mg of protein in the different samples. In xtranuclear DNA polymerase activity in the soluble and cytoskeletal fractions of dividing and non-dividing cells.

	Activity in CSF + soluble fractions[a]						
	Per mg protein		in 1 gm tissue[b]		in 10^8 cells[c]		% (of CSF + cytoplasmic) activity in CSF
Sample source	sol[d]	CSF[d]	sol	CSF	sol	CSF	
Adult liver	1·9	6·5	131·1	69·0	437·1	230·9	34·5
Regenerating liver	6·5	2·8	375·9	22·7	1253·3	75·5	5·6
Fetal liver	11·2	1·8	670·0	33·0	670·0	33·0	4·7
ZAH cells	18·1	2·9	1740·0	54·0	870·3	28·6	3·1

[a] Activity expressed as *p* mol of $[^3H]$-TMP incorporated in 15 min. reaction using activated calf thymus DNA as template. Values reported are averages of 3 separate determinations in each case.

[b] Calculated from net weight of starting tissue.

[c] Adult liver and regenerating liver have 3×10^7 cells/g tissue whilst fetal liver has 1×10^8 cells/gm tissue and ZAH has 2×10^8 cells/g tissue.

[d] sol, soluble fraction.

Discussion

We have demonstrated biochemically the presence and characteristic distribution of DNA polymerase-α in the soluble and CSFs of resting and dividing cells. These observations are in sharp contrast with most immunocytochemical studies indicating an exclusive nuclear location for DNA polymerase-α. As the enzyme must be synthesized in the cytoplasm, it is surprising that no enzyme can be detected at the site of synthesis by immunocytochemical methods. It is therefore likely that these methods especially those making use of monoclonal antibodies may be detecting specific epitopes, which may be masked in the enzyme present outside the nucleus.

Differences in the state of association of DNA polymerase-α with nuclear matrix in resting and dividing cells have been documented (Smith and Berenzney, 1982). In the present experiments we demonstrate a differential association of the enzyme with CSF in resting and dividing cells. Such an association and possibly a functional role of the association is not surprising in light of the increasing realization of the role of CSF in a variety of cellular events. It is likely that the cytoskeletal framework may play a role in the transport of DNA polymerase-α within the cell. When the cells are dividing, any transport of the enzyme through the mediation of the skeletal framework could be more active and the steady state concentration of the amount of enzyme remaining associated with CSF could be lower. In the resting phase, part of the enzyme synthesized at the time of division could remain associated with skeletal elements probably better protected, to be readily available at the onset of cell division.

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